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BACTERIOLOGY

IN

ABSTRACT

WALLGREN

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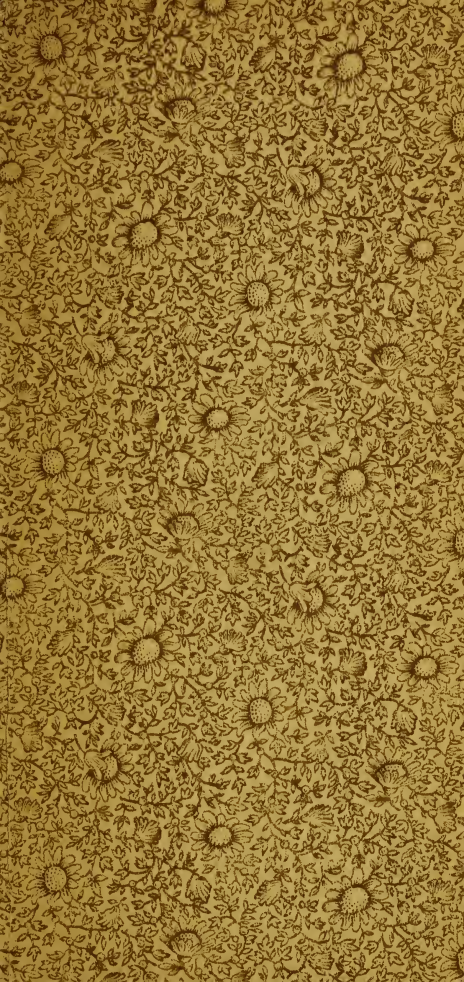


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BACTERIOLOGY IN ABSTRACT

BY

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PREFACE TO SECOND EDITION

The demand created by the first Abstract in Bacteriology necessitated the production of a second edition, which, while considerably enlarged, contains only the very elements of Bacteriology. Although containing only the first principles of Bacteriology, it is hoped that this book will be of use to the student wishing a small work for a ready guide.

For a detailed work the student must necessarily refer to the works of Jordan, Mallory and Wright, Chester, Frost, Frost and McCampbell, Marshall, Park and Williams, Hiss and Zinsser, Besson, Gorham, Muir and Ritchie, Simon, etc., all of which have been freely consulted in the preparation of this Abstract.

The Author is very much indebted to Eleanor C. Doty for valuable aid in the preparation of manuscript and the reading of proofs.

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HISTORY OF BACTERIOLOGY.

With the introduction of the microscope some order was brought into understanding that group of organisms which Linnaeus had termed "chaos." That disease and decay were due to minute organisms had been the theory for centuries. The conception of contagion, or the transmission of disease from one human being to another, was centuries old. This fact had been recognized by Aristotle and had been reiterated by medieval philosophers, and had led to the division of contagious diseases, by Fracastor (1546), into those diseases transmitted "per contactum" and those conveyed indirectly "per fomitem." It was on account of these facts of transmissibility of disease that physicians of the eighteenth century saw an explanation in the microorganisms discovered in the latter half of the seventeenth century by the Jesuit, Kircher (1659), and the Dutch linen-draper, van Leeuwenhoek (1675). These two men were able by the improved microscope to demonstrate microorganisms in water, intestinal contents, etc. They made out short, straight, and curved rods, and described their motility. There can be no doubt that the bodies seen by these two men were, at least in part, bacteria. During the century following the work of these two investigators, a more exact description of these forms of life led Muller to attempt a systematic classification. A more extensive study and classification was later made by Ehrenburg.

Needham, in 1749, published an article in which he favored the opinion, held by many, namely, that the minute organisms described by Leeuwenhoek and others were produced by spontaneous generation. He held to this opinion from the fact that he had placed putrefying material and vegetable infusion in sealed flasks, exposing them for a short time to heat, and later found this infusion to be filled with microorganisms. Buffon supported him in his views. Abbe' Spallanzanni repeated the experiments of Needham, employing greater care in sealing his flasks, and subjecting them to a greater exposure of heat. His results were un-

like those of Needham and consequently did not support the theory of spontaneous generation. Schulze's (1836) failure to find a living organism in infusions which had been boiled and to which air had been admitted only after passage through acid solutions, did not convince many that spontaneous generation was impossible, and that the life that appeared in the infusions was due to the presence of minute organisms having entered the infusion by reason of faulty technique. The question of spontaneous generation was not definitely settled until Pasteur (1860) conducted a series of experiments, the results of which absolutely refuted the doctrine of spontaneous generation.

Plenciz of Vienna (1762), expressed his belief in the direct etiological connection between microorganisms and certain diseases, and also suggested specific treatment for these diseases.

Rayer, in 1815, found rod shaped organisms in the blood of animals sick with splenic fever. The real advance in the development of bacteriology came in 1837 when Schwann showed that yeasts were living organisms and had to do with the process of fermentation. The same view was held by the Frenchman Cagniard-Latour.

Pasteur paralleled his researches upon spontaneous generation with experiments upon fermentation along the lines suggested by Cagniard-Latour, publishing his classical studies upon the fermentation that occurred in beer and wine, due to yeasts, and was also able to show that a number of other fermentations, such as those of lactic and butyric acid, as well as the decomposition of organic matter by putrefaction, were directly due to the action of microorganisms. The doctrine of spontaneous generation had received its final refutation in all but one particular. It was not understood why sterility could not always be obtained from the application of definite degrees of heat. This was finally explained by Cohn (1871), who demonstrated the presence of bacterial spores, also demonstrating their resistance toward heat and other influences.

Pollender, in 1855, reported the presence of rod shaped bodies in the blood

and spleen of animals dead of anthrax, previously reported by Rayer. Convincing proof of the observations of Rayer and Pollender was brought out by Davaine (1863), who also succeeded in demonstrating that anthrax could be transmitted by means of blood containing the rods described by Rayer and Davaine, and could never be transmitted by blood from which the rods were absent. Anthrax, therefore, was the first disease in which bacterial causation was demonstrated.

Obermeier (1868) demonstrated the presence of a spirillum in the blood of patients suffering from relapsing fever.

Rindfleisch, von Recklinghausen, Waldeyer described microorganisms in tissues containing abscesses.

Klebs (1870) described similar microorganisms found in pus.

Koch (1880) introduced nutrient media upon which bacteria could be cultured, thus laying the foundation for an exact science.

Weigert, Koch, and Ehrlich introduced the use of aniline dyes which facilitated the morphological study of bacteria.

Koch published the discovery of the typhoid bacillus, fowl cholera bacillus and pneumococcus in 1880 and the tubercle bacillus in 1882.

THE BIOLOGY OF BACTERIA.

ORIGIN, DISTRIBUTION, ETC.

Bacteria cannot arise *denovo*. They must develop from pre-existing bacteria or their spores. One kind of organism will not produce another kind.

DISTRIBUTION.

Air. In the air there are bacteria throughout, except at great altitudes.

Soil. In soil, bacteria are very abundant except at great depths. Garden soil will contain from a hundred thousand to as many millions per gm. Bacteria are less numerous in the peat or acid moor soils, in the soils containing an excess of alkali and in sandy soils. Were it not for bacteria, plant life could not exist as they perform functions in the soil,

fitting it physically and chemically for the growth of higher plants. They decompose organic matter directly, and minerals of the soil indirectly, converting them into compounds that are taken up and assimilated by plants, etc.

Water, as a rule, is the home of bacteria.

Wells contain from a very few to several hundred bacteria per c. c., though in deep wells, such as artesian, and in the springs of some districts, there may be no bacteria. There are about the same number of bacteria in lakes and ponds as in wells, except where there is sewage contamination.

Streams usually contain more bacteria than any of the above by reason of sewage contamination or washings from water sheds, etc.

Foods. Bacteria are most abundant in most foods. Some healthful foods contain great numbers of bacteria, as is illustrated by the lactic acid bacteria of sour milk.

Other bacteria present in food, in great numbers, may be harmless.

The bacteria of foods may be divided into three groups:—

- (1) Those beneficial—which bring about the fermentations, as in the preparation of pickles, sauerkraut, etc.
- (2) Those producing fermentations and decay.
- (3) Those producing disease.

Fermentation bacteria act upon carbohydrates, as starch and cellulose, breaking them down. Decay-bacteria decompose proteins.

Body. The normal living tissues of the body are free from bacteria.

Bacteria are found in great numbers on the skin, in the alimentary tract, and can be considered as a flora peculiar to this situation as they ordinarily do no harm. Certain bacteria (pathogenic) may enter the body and produce disease.

MICROORGANISM NORMAL TO THE HUMAN BODY.

A great number of species of bacteria develop on the skin of the body and

in the various body cavities which open to the surface. Normally the tissues of the body are sterile. Bacteria are not found in the blood stream, in the muscles, nor in the glands of normal individuals although the lymph fluids lying near the intestines may occasionally be infected by the intestinal bacteria. Organisms may or may not be present in the tissues of the individuals. Some diseases are classified as septicemias and bacteriemias because of the general distribution of the bacteria in the blood stream. In other cases, the organisms are localized and do not get far from the site of infection.

The exposed surfaces of the body and the cavities of the body that communicate with the surface possess a bacterial flora that may be considered normal to the part.

The Flora of the Skin.

The organisms that are constantly present and evidently multiply upon the surface:—

Micrococcus (staphylococcus) *epidermidosis albus*, an organism which is closely related to the pus producing cocci. This organism frequently penetrates into the lower layers of the epidermis.

Micrococcus aureus and *albus* are frequently present, but are usually of a low virulence. These are the organisms that produce infection of wounds, abscesses, and boils.

Streptococci may frequently be isolated from the skin.

Bacterium smegmatis is nearly constantly present in places where the skin is moist and greasy. It does not seem to penetrate into the skin but lives upon the skin excreta.

In the wax secreted in the external auditory canal, the *micrococcus cereus flavus* is generally found.

Very few bacteria are found normally on the conjunctiva. The tears seem to remove or destroy any organism that may gain entrance.

The organisms that accidentally form a skin flora are the bacteria that contaminate the skin when it comes into contact with dust or dirt. The soil bacteria are quite common to the skin, particularly under the

finger nails and in the hair. These include the soil aerobic forms, as the bacillus subtilis group and certain anaerobic bacteria.

The intestinal organisms, as the bacillus coli, are commonly present by reason of contact with diseased individuals.

The Flora of the Mouth.

A great number of the organisms that have been isolated from the mouth are probably due to accidental contamination. Mueller has described a number of species found present in the normal mouth. Of these, he decided that six were constantly present; i. e., leptothrix and the spirocheta dentium. These organisms are obligative anaerobes and find favorable conditions for growth between the teeth and in teeth undergoing decay.

Anaerobic bacilli related to *B. putrificus*.

Non virulent varieties of streptococcus pyogenes.

Non virulent varieties of micrococcus aureus and albus.

Avirulent pneumococcus.

The decay of teeth is said to be due to the development of acids by certain lactic acid bacteria present upon the surface, causing a more or less complete removal of the lime present; while the decomposition of the remaining substance of the teeth is probably brought about by common anaerobic bacteria. Occasionally the mouth of a healthy individual may contain pathogenic organisms, as the bacterium diphtheria and bacterium influenza.

The Flora of the Stomach.

Ordinarily bacteria do not develop in the stomach by reason of its acidity. If for any reason the acidity is diminished, bacteria belonging to the butyric acid group and the bacterium lactis aerogenes may develop. Under certain conditions, the lactic acid bacteria of the Bulgarian type may develop.

Flora of Intestine.

The first portion of the intestine is relatively free from bacteria by reason of the fact that the bile is antiseptic to certain microorganisms

and will, more or less, completely inhibit their growth. In the lower part of the small intestine bacteria become more numerous. The bacterium *lactis aerogenes* and *bacillus coli* are present in small numbers. In the colon, bacteria develop freely. The bacterium *lactis aerogenes* and the *bacillus coli* are nearly always present. These together with the *bacillus bifidus* and *bacillus acidophilus* inhibit the growth of any organism of the putrefactive type by the formation of an acid, and possibly by the formation of metabolic products. Anaerobic forms, as the *bacillus putrificus* and *bacillus aerogenes capsulatus*, are also present.

It is quite possible that in some of the herbivorous animals the organisms are of some assistance in the digestion of food. In the human intestine it is believed that products of decomposition brought about by the putrefactive bacteria are absorbed through the intestinal wall and appear in the same form or as related compounds in the urine, and cause some of the changes characteristic of old age, particularly the hardening of the arterial wall (arterial sclerosis).

Metchnikoff believed that the putrefactive bacteria might be eliminated by using foods containing great numbers of lactic acid bacteria, which would prevent the development of the putrefactive form by the presence of the lactic acid bacteria and also the lactic acid.

The Flora of the Respiratory Tract.

The flora of the upper respiratory passages, as the nose, can not be said to be characteristic. The bacteria isolated from the nasal cavities are generally the bacteria of the air. The air of the bronchi, the bronchioles and alveoli of the lungs are usually entirely free from microorganisms by reason of the filtration of air through the nasal passages, and the passage of the air over the nasal and oral mucous membrane, which is covered with mucous and also with ciliated cells, which serve to some extent to wash

the mucous containing the bacteria from the surface. Infections of the nasal mucous membrane are, however, not uncommon. The bacterium influenza, the streptococcus pyogenes, the micrococcus pyogenes, aureus and albus, bacterium diphtheria, the micrococcus intracellularis, meningitidis and occasionally the bacterium mallei produce infection through the membrane of the nasal cavities.

The Flora of the Genito-Urinary Tract.

The exposed mucous surfaces of the genito-urinary tract usually harbor a number of harmless bacteria. Organisms closely related to the pus producing cocci are commonly present. The secretions of the vagina are somewhat bactericidal, but certain bacteria are constantly present and the properties of the vaginal secretion is supposed to be in part due to the presence of these organisms, e. g. Doederlein's bacillus.

Normally the uterus does not contain bacteria, although their presence is not uncommon in the later stages of pregnancy. The urinary bladder is normally free from bacteria.

In the secretions about the external genitals, acid-fast bacteria, particularly the bacterium smegmatis, are constantly present.

THE CATABOLIC ACTIVITIES OF BACTERIA

Study of substances that result from the action of the life of bacteria and the changes that they produce in the various media of growth, is really a branch of organic chemistry. So we can only make mention of them here. The function of bacteria is essentially a destructive one. They split up the higher nitrogenous and non-nitrogenous compounds into simple substances.

The various substances that are found in the media of bacterial growth **comprise:—**

1. The components of the bacterial cell proper, as the proteins.
2. The secretions of the cell, as the ferments and toxins.
3. The substances that are the result of the action of the organ-

isms upon the medium of growth:—

The toxic substances in bacterial cultures may be classified as (a). Intracellular and (b) Extracellular, according as they are contained within the bacterial cell or free in the culture medium. The extracellular substances may be purely products of bacterial secretion which have separated from the cell or they may be decomposition products derived from the culture medium.

- (1) **The proteins** may produce suppurations or fever, or may cause inflammatory processes. They are comparatively resistant to heat and are thus sharply distinguished from the ferments and toxins. The best known examples are mallein, derived from the bacillus of glanders, and tuberculin from that of tuberculosis. These substances are **pyogenic** (fever) when injected into animals sick with glanders or tuberculosis but have very slight effect upon healthy subjects.
- (2) Second group are ferments and possibly toxins.

Ferments are complex bodies about which very little is known, except the effects they produce. By their presence and probably without entering into intimate chemical combination, they possess the power of breaking up more highly organized nitrogenous and non-nitrogenous compounds into simple and more diffusible molecules.

They are termed **enzymes** or **unformed ferments** in contradistinction to the bacteria themselves. That the action of ferments is not due directly to the organism is shown by the fact that bactericidal substances, such as phenol 5%, chloroform, ether, etc., have no effect on them and that cultures freed from bacteria by filtration still possess fermentative power.

The action of ferments is termed **fermentation**, but this term is more especially limited to the effect of certain ferments upon non-nitrogenous compounds, particularly the carbohydrates. The result of fermentation upon nitrogenous material is called **putrefaction**, which generally occurs with, though often without, the formation of odorous gases and other substances.

The intracellular origin of certain ferments has been demonstrated by their experimental separation from the bacteria when placed under high pressures. The resulting bacteria-free liquid possesses the same fermenting qualities as the culture itself.

Ferments like toxins are of unknown composition, are highly destructible by chemical agents and heat, and cause effects out of all proportion to their bulk or amount. They are frequently mechanically precipitated with various indifferent bodies. When injected into animals both are capable of exciting the formation of antibodies (anti-ferments and antitoxins).

The principal ferments are:—

Proteolytic ferments. Transforming albumins into more soluble and diffusible substances.

These ferments find their analogy in the ferments of the stomach and pancreas.

They digest the various albumins with the formation of albumoses and those end products of hydrolysis which are collectively spoken of as peptones.

One form very often met with liquefies gelatin. It acts in an alkaline medium and is therefore akin to the animal ferment trypsin. This liquefaction of gelatin affords a means of distinguishing many species of organisms.

Amylolytic ferments (amylasis diastases) transform starches into sugar and are found in many bacterial cultures, e. g.,

B. mallei, *B. pneumonia*, etc. They find their analogy in the secretions of the pancreas and the ptyalin of saliva.

Inverting ferments (Invertases.)

They are apparently related to the amylolytic ferments and are to a certain extent identical with them. They invert disaccharides to monosaccharides and according to their specific action upon cane sugar, maltose and lactose, they are termed — invertases, maltases and lactases, respectively. They have their analogies in the saliva and pancreatic juice. Such ferments are found in cultures of *spirillum cholera* and *Metchnikovi*.

Emulsifying Ferments are formed by but few bacteria. One example is *micrococcus pyogenes tenuis*.

Coagulating Ferments. In these we have a means of differentiating bacteria by their coagulation of milk. This coagulation is not due to acids produced in the medium but to the action of a ferment. Some varieties of bacteria produce a ferment that has the power of dissolving this coagulum when formed (casease). Other bacteria produce both ferments—the coagulating and the dissolving.

Lipolytic Ferments. Fat splitting ferments (lipases).

They cause hydrolysis of fats into fatty acids and glycerin. They have their analogues in the pancreatic and gastric juice.

Ureases (hydrolytic) break up urea into ammonium carbonate and hippuric acid into glycolic and benzoic acid. They are in such bacteria as the *micrococcus urea*, *bacterium urea*, *bacillus fluorescens*, etc.

Oxidases. Produced by several bacteria, especially the chromogens. They act upon complex organic compounds changing them to colored bodies,

Reductases. This ferment is contained by all but a few bacteria, among the exceptions being the *Bact. acidi lactici*. They will reduce nitrates to nitrites, sulphur to hydrogen sulphide, etc.

Effects of Ferments. The single or combined action of these various ferments cause certain kinds of fermentation distinguished by the principal substance produced. Alcoholic, lactic acid, and butyric-acid fermentation of the sugars, acetic acid fermentation of alcohol (*B. acidi lactice*, *B. butyricus*, *B. acidi butyrici*, *B. aceticus*, etc.); cellulose fermentation with the production of carbonic acid gas and ammonia; nitrification, in which oxidation of ammonium leads to the production of nitrites and secondarily, conversion of nitrites into nitrates; mucoid fermentation of glucose and invert sugar are examples.

Toxins will be taken up later.

The study of those substances resulting from the actions of the life of bacteria in the media of growth, is accomplished by the so-called

Biochemical Methods, i. e.,

Test the cultivations of the organism for the presence of—

1. **Soluble enzymes**—proteolytic, diastatic, invertase.
2. **Organic acids**—(a). Quantitatively—i. e. Estimate the total acid production. (b). Qualitatively, for formic, acetic, propionic, butyric and lactic.
3. **Ammonia**.
4. **Neutral Volatile substances**—ethyl, alcohol, aldehyde, acetone.
5. **Aromatic products**—Indol—phenol.
6. **Soluble pigments**.
7. **Reducing Powers** (a) coloring matters, (b) nitrates to nitrites.
8. **Gas production**— H_2 , $S.$, CO_2 , $H.$ Estimate the ratio between the last two gases.

Prepare all cultivations for these methods of examination under optimum conditions, previously de-

terminated for each of the organisms it is intended to investigate as to—

- (a) Reaction of medium.
- (b) Incubation temperature.
- (c) Atmospheric environment.

Keep careful records of these points, also the age of the cultivation used in the final examination.

Examine the **cultivations** for the various products of bacterial **metabolism** after 48 hours growth, and **never omit** to examine "**control**" (uninoculated) **tube** or **flask** of medium from the **same batch**, kept for a similar **period under identical conditions**.

If the results are negative, test further cultivations at 3, 5 and 10 days.

CHEMICAL CONSTITUENTS OF THE BACTERIAL CELL.

The chemical composition of bacteria varies with their food supply.

About 85% of the bacterial body is water.

The remainder is chiefly proteids which constitutes from 50 to 85% of the dry substance. After the proteid material has been extracted there are fats and in some instances true wax (fatty acid combinations with higher alcohols. In traces of cellulose and ash the ash constitutes about 1 to 2% of the dry substance, and is made up largely of phosphates and chlorides of potassium, sodium, calcium and magnesium.

NUTRITION OF BACTERIA.

In order that bacteria may develop and multiply, they must be supplied with the substances of which they consist, in proper quantity and in forms suitable for assimilation, namely, carbon, oxygen, hydrogen, nitrogen, inorganic salts and varying quantities of phosphorus and sulphur.

Carbon is necessary for their nourishment, and may be obtained from proteids, carbohydrates and fats, or from their derivatives.

Oxygen. Free oxygen is necessary for the growth of many bacteria (obligatory aerobes). For these it is obtained directly from the atmosphere in the form of free O. Another class of bacteria is unable to develop in the

presence of free oxygen (obligatory anaerobes), and they obtain their oxygen indirectly by the enzymatic processes of fermentative and proteolytic cleavage, from carbohydrates and proteids, or by reduction from reducible bodies.

There is still another large group of bacteria which develops well under both aerobic and anaerobic conditions. Some of these have a preference for free oxygen, but will thrive without it (facultative anaerobes). In others the reverse is true (facultative aerobes.)

Nitrogen is in most cases obtained from proteids. The diffusible proteids are the most important, but many of the non-diffusible albumins may be rendered assimilable by the proteolyzing enzymes possessed by many bacteria.

A large number of bacteria may develop on media containing no proteid, in which case the organism produces a synthetic proteid.

Many bacteria may obtain their nitrogen from creatin, creatinin, urea and urates and ammonia compounds and nitrates.

A few bacteria, as the bacilli in the root tubercles of legumins and the nitrogen-fixing bacteria of the soil, obtain their supply of nitrogen directly from the free N of the air.

Hydrogen is obtained in combination as water and together with the carbon and nitrogen containing substances.

Salts. The phosphates are necessary constituents of culture media and are taken in as phosphates of magnesium, calcium, sodium or potassium.

The chlorides are not absolutely essential (Proskener and Beck).

The sodium salts seem better for purposes of cultivation than potassium salts.

The sulphur is usually taken in as soluble sulphates. The thiobacteria of Winogradsky demand free H_2S .

The iron in the higher bacteria is taken in as ferrous compounds and is oxidized in the bacterial body into ferric compounds.

BIOLOGIC ACTIVITIES OF BACTERIA.

Without the bacterial processes, which are constantly active in the reduction of complex organic substances to their simpler compounds, the chemical interchange between the animal and vegetable kingdom would fail and all life would cease.

They are paramount factors in the cycle of living matter supplying the links in the constant circulation of nitrogen and carbon compounds necessary between the plant and the animal kingdoms, through their anabolic or constructive process in the one and their catabolic or destructive process in the other.

The catabolic activities of bacteria consist in the fermentation of carbohydrates and in the cleavage of proteids and fats (see ferments or enzymes; also denitrifying bacteria in nitrogen cycle).

THE ANABOLIC OR SYNTHETIC ACTIVITIES OF BACTERIA.

The depletion of the soil by constant withdrawal of nitrogenous substances by plants would soon be complete, were it not for certain forces constantly at work replenishing the supply from the free nitrogen of the air. Bacteria, to a large extent, return nitrogen to the soil (see "Nitrogen fixation," also "Nitrification" in nitrogen cycle).

Light Production by bacteria, is seen in certain salt water forms. Much of the phosphorescence observed at sea is caused by bacteria. They are closely allied to the putrefactive bacteria and in the sea are usually found upon rotting animal matter.

The light production is dependent upon free access of oxygen and their luminous quality is not a true phosphorescence in that it does not depend upon previous illumination.

The formation of pigment by bacteria is also a result of anabolic activity.

ANALYTIC CHANGES PRODUCED BY MICRO-ORGANISMS.

It is the purpose to briefly outline the changes induced, with particular

reference to the so-called cycles of certain of the elements and the part played by microorganisms in inducing these changes. The most important elements to be considered are nitrogen, carbon, sulphur and phosphorus.

1. **The Cycle of Nitrogen in Nature.**

Nitrogen is found in nature in 3 principal forms:—Free in the air, as a gas; in inorganic compounds such as ammonia, nitrites, and nitrates; and in organic compounds.

Microorganisms are important in changing nitrogen from one form or combination to another; in fact, without their activity, it would be impossible for higher animals and plants to exist on the earth. It will probably be most convenient to start with complex organic nitrogenous compounds in the study of the nitrogen cycle and the changes to be considered are:

- (a) **Ammonification**, which is the conversion of complex nitrogenous compounds into simpler forms, and ultimately into ammonia.

This occurs in several distinct stages. The complex protein molecule is broken up into somewhat simpler compounds—the proteoses, and then into peptones; (termed peptonization). These peptones are broken down by various organisms with the formation of polypeptids and amino acids primarily, and a considerable number of secondary products.

The amino acids are still further decomposed with the production of ammonia.

The whole process of successive cleavages of proteins is sometimes termed proteolysis. The principal nitrogenous waste product of the decomposition of proteins in the body is urea. This is actively transformed by certain bacteria into ammonium carbonate. It is evident that all organic nitrogenous compounds are ultimately reduced to ammonia by the process of **Ammonification**.

This is of very great economic importance in agriculture, as nitrogen in this form is readily changed so as to become available to higher plants.

The various steps in ammonification may be brought about by different organisms. A few species can attack native proteins; many, however, can utilize and change only the peptones, the peptids and amino acids. The organisms changing urea to ammonium carbonate constitute a very distinct group.

- (b) **Nitrification.** (Nitrifying Bacteria). Certain microorganisms, common in the soil, particularly a coccus (nitrosococcus), are able to oxidize ammonia to **nitrous acid**. They secure their energy for growth by this change. The process is termed **nitrosation**. Normally, the nitrous acid is neutralized, at once, by the bases of the soil thus forming nitrites. These nitrites soon undergo the next change, **nitration**, or oxidation to nitrates by other species of soil bacteria. The nitrates, and to a less degree the ammonia, constitute the source of nitrogen for higher plants.

No nitrogenous manure is effective in increasing crop yield that is not capable of being ammonified and nitrified.

- (c) **Nitrogen Assimilation** is in large part a function of the higher plants.

The nitrates, and to a less degree the ammonia, produced by bacterial activity in the soil are taken up through the roots and built up into protoplasm and complex proteins.

These may decay or they may be eaten by animals, but ultimately they are decomposed by microorganisms.

This alternate synthesis of proteins by higher plants and disintegration by microorganisms constitutes the principal part of the nitrogen cycle.

- (d) **Denitrification.** Nitrates, in the absence of oxygen and in the presence of organic matter, may reduce to nitrites by bacterial activity, and these nitrites further decompose with liberation of free nitrogen. Under these conditions, microorganisms take the oxygen from the molecule of nitrate or nitrite. Some species, for example, will live under anaerobic conditions if nitrates are present, otherwise they are aerobic.

This fact is of some significance in agriculture in explaining loss of fertility in water-logged soils.

- (e) **Nitrogen Fixation.** If gaseous nitrogen is lost from the cycle as a result of denitrification, there must be some method whereby it can be again fixed or combined.

A certain number of species among the bacteria and moulds are known to possess this power.

Certain of the higher plants have mould-like fungi which live upon their roots and take up the atmospheric N (e. g. Alders, Russian olives, and certain other trees, the orchids and many plants living in peat bogs and swamps).

These organisms are termed **mycorrhizas**.

The *B. radicicolus*, a minute bacterium, produces nodules or tubercles on roots of many leguminous plants as the bean, pea, olive and alfalfa. These swellings are found to be made up of cells tightly packed with bacteria.

These organisms take N from the air and directly or indirectly transfer it in part to the host plant. Legumens, unlike most plants, therefore, can grow in soil devoid of N., provided the roots are supplied with nodules. The efficiency of legumens in increasing the fertility of the soil is due to this fixation of nitrogen.

There are also a few living soil bacteria which can take up nitrogen from the air. These belong to two groups, anaerobes and aerobes; some spore bearing soil bacilli (*Clostridium*), in the presence of proper food, such as certain carbohydrates, can fix some nitrogen under anaerobic conditions. These forms are not very important in the soil.

Much more important are the aerobic **Azotobacter**. These secure energy for the fixation of nitrogen by the oxidation of carbohydrates. They are probably very important in soil fertility.

2. **Carbon Cycle.** This cycle in nature as affected by microorganisms is more simple than that of N.

It is well here to emphasize two facts:— (1) All plants and animals alike are continuously developing CO_2 ; (2) Some plants can synthesize organic compounds, principally carbohydrates and fats, from CO_2 .

All active cells are constantly breaking down carbon compounds; some can also build them up. All plants containing chlorophyll or leaf-green use CO_2 and water to produce starch and sugars, gaining the energy necessary by means of the absorption of sunlight. A few bacteria containing bacterio-purpurin are also capable of using light for this purpose. Some forms oxidize ammonia to nitrites, nitrites to nitrates, H_2S to sulphur or sulphur to H_2SO_4 , and utilize the energy thus secured in building up food materials.

The carbon cycle, then, consists of the alternate building up of carbon into organic compounds and their subsequent disintegration with ultimate oxidation of the Carbon to CO_2 .

3. **Sulphur Cycle.** The decomposition of organic compounds containing sulphur usually results in the evolution of H_2S . This

is readily oxidized by many aerobic bacteria with the production of free sulphur and sulphuric acid. These organisms are abundant in sewage and in water of sulphur springs. In these springs, they may form masses of considerable size. The sulphur granules may be seen within the cells of the organism.

Reduction of Sulphur Compounds with formation of H_2S occurs when sulphates in the presence of organic matter are subjected to anaerobic conditions.

The sewage of some cities is very offensive because the city water contains sulphates in considerable quantity. Bacteria cause decomposition of organic matter of the sewage reducing the sulphate and the sulphite is formed.

Phosphorus and Calcium Cycles in nature show a change which is influenced by microorganisms.

CLASSIFICATION OF BACTERIA.

Involution, Structure, Reproduction, Biological Classification.

Bacteria are minute unicellular organisms which may occur free and singular, or in larger or smaller aggregations, thus forming multicellular groups or colonies, the individuals of which are, however, physiologically independent. They occupy the lowest plane of plant life. The position which they occupy in plant life is shown below:—

Plants.

A. **Cryptogamia** (flowerless plants forming spores).

1. **Pteridophyta**. e. g. ferns, horse-tails and club mosses.
2. **Bryophyta**. e. g. liverworts and mosses.
3. **Thallophyta**. e. g.
Myxomycetes (slime-fungi).
4. **Schizophyta** (fission plants)
Schizophyceae (fission algae)
Schizomycetes (fission fungi
or **Bacteria**).

Diatomea (diatomis), Chlorophyceae (green algae).
 Rhodophyceae (red algae),
 Phaeophyceae (brown algae).
 Characeae (stone worts), Hy-
 pomycetes (fungi).

Lichens.

B. **Phanerogamia.** (flowering plants, forming seeds).

Bacteria, Schizomycetes, are classified by Migula into:—

1. **Eubacteria,** cells contain no sulphur granules or bacteriopurpurin.

1. Family Coccaceae, spherical forms

Genus:

(a) Streptococcus, non-motile; cells divide in one plane.

(b) Micrococcus, non-motile, cells divide in two planes.

(c) Sarcina, non-motile; cells divide in three planes.

(d) Planococcus, motile; cells divide in two planes.

(e) Planosarcina, motile; cells divide in three planes.

2. Family Bacteriaceae, straight, rod-shaped forms without envelope.

Genus:

(a) Bacterium, non-motile.

(b) Bacillus, motile; flagella over whole surface.

(c) Pseudomonas, motile; flagella polar.

3. Family Spirillaceae, curved rod-shaped forms without envelope.

Genus:

(a) Spirosoma, non-motile; cells rigid.

(b). Microspira, motile; one, rarely two or three polar flagella.

(c) Spirillum, motile; polar tufts of flagella.

(d) Spirochaeta, cells flexible.

4. Family Chlamydobacteriaceae, cells with envelopes.

Genus:

(a) Chlamydothrix, unbranched threads; cell-division in one plane; (b) crenothrix unbranched threads; cell

division in three planes;
sheath visible.

(c) *Phragmidothrix*, unbranched threads; cell-division in three planes; sheath scarcely visible.

(d) *Sphoerotilus*, branched threads.

II. Thiobacteria, cells contain sulphur granules or bacterio-purpurin; red or violet color, never green.

1. Family *Beggiatoaceae*, thread-forming, without bacterio-purpurin.

Genus:

(a) *Thiotrix*, attached threads; non-motile.

(b) *Beggiatoa*, unattached threads; motile.

2. Family *Rhodobacteriaceae*, cells contain bacterio-purpurin and sulphur granules; red or violet.

A. Subfamily *Thiocapsaceae*, cells divide in three planes.

Genus: *Triocystis*. *Thiocapsa*. *Thiosarcina*.

B. Subfamily *Lamprocystaceae*, cells divide first in three, then in two planes.

Genus: *Lamprocystis*.

C. Subfamily *Thiopediaceae*, cells divide in two planes.

Genus: *Thiopedia*.

D. Subfamily *Amebobacteriaceae*, cells divide in one plane.

Genus: *Amebactor*. *Thiothece*. *Thiodictyon*. *Thiopolycoccus*.

E. Subfamily *Chromatiaceae*.

Genus: *Chromatium*. *Rhabdochromatium*. *Thiospirillum*.

A commonly used classification subdivides bacteria into:—

I. Lower Bacteria, which are microscopic in size, multiply by fission and contain no chlorophyll.

1. **Cocci**, are globular in form.

(a) Single coccus.

(b) Diplococcus.

(c) Staphylococcus.

(d) Streptococcus.

(e) Tetrads.

(f) Sarcina.

2. **Bacilli**, are straight rods.

(a) Long.

- (b) Short.
- (c) Diplobacillus.
- (d) Irregular.
- 3. **Spirillae** are curved or spiral rods.
 - (a) Comma.
 - (b) Spiral.

II. Higher Bacteria, have a more complex organization. They consist of filaments built up of separate individuals, some of which seem related to physiologic labor and some seem for the purpose of reproduction.

They possess the following characteristics: They are attached, unbranched, filamentous forms, showing a differentiation between base and apex; growth apparently apical; exaggerated pleomorphism; pseudo branching from opposition of cells and are classified into—*Beggiota* and *thiothrix*; free swimming forms, which contain sulphur granules.

Crenothrix, *cladothrix* and *leptothrix* do not contain sulphur granules.

Streptothrix; a group which exhibits true but not dichotomous branching and contains some pathogenic species.

Branched forms (normal though unusual) must not be confused with involution forms. They are divided into:—

1. **True branching**—a bud springs out from the bacteria, e. g. *Bacillus tuberculosis*, and the bacillus of diphtheria.
2. **Dichotomous**, which is often confounded with true branching. It is, however, a misnomer, as it means a branching in two equal parts.
3. **Pseudodichotomous**, or false branching is due to the opposition of separate organism. The streptococci may produce false branching by one cocci dividing at right angles to chain and in this way producing a new chain of cocci which branches from the original chain.

INVOLUTION FORMS OF BACTERIA.

Degeneration Forms or Pleomorphism.

Bacteria grown on artificial media, or having grown in the same media for some time; i. e. under conditions not favorable for their growth may show abnormal or unusual shapes (pleomorphism).

Involution forms characterized by alterations of shape are not necessarily dead, but those forms characterized by a loss of staining power are always dead.

STRUCTURE.

Cell membrane or **Capsule** is a dense, highly refractile, gelatinous outer portion or covering of the cell wall of some bacteria. It will absorb moisture and swell. Organisms having a capsule, when in suitable solution, make the solution gelatinous or slimy. This condition gives rise to slimy bread and ropy milk.

The composition of the capsule may be nitrogenous or non-nitrogenous. Substances such as mucin, mannans, galactans and dextrans have been identified.

An organism may produce a capsule under certain conditions only, as in the blood, urine or milk, but not in most culture media.

The membrane prevents certain bacteria, such as the streptococcus and the staphylococcus, from becoming separated, forming them into chains or bunches.

Bacteria growing in gelatinous masses, secreted by the cell, is known as **Zoogloea**. It can be seen in sewage and on filter beds.

The capsule is not easily demonstrated by the ordinary staining methods.

Cell wall lies between the capsule and the cell protoplasm, from which it is modified. Its chemical composition differs in different bacteria.

In some bacteria it is of a cellulose reaction in others an albumin, and in chemical composition it resembles the chitin of the lower invertebrates. All the food passes through it by

diffusion, it having no selective power. It can be easily demonstrated.

Cell content is mainly protoplasm, composed of mycoprotein. As a rule it is homogeneous, but may contain granules, fluid spaces, fat droplets, pigment, sulphur and chlorophyll.

That portion next to the cell wall, called **ectoplast**, is an important structure as it has to do with nutrition. In many bacterial cells it is semipermeable, allowing some substances to pass through, inhibiting others. A demonstration of its action as an osmotic membrane may be had by placing certain bacteria in strong sugar solutions, causing the protoplasm to shrink (**plasmolysis**).

A definite nucleus has not been demonstrated, though the granules present are probably nuclear in nature. Their behavior during cell division would probably indicate them to be a primitive nucleus.

Metachromatic granules (e. g. diphtheria) derive their name from their ability to take up basic aniline dyes, as does chromatin.

Sheath. Certain bacteria growing in a chain secrete a firm membrane (sheath) in such a way as to form a tube in which the organism lives. e. g. *Chlamydothrix*, *Crenothrix* and *Cladothrix* substances, such as iron or calcium compounds, may be deposited in this sheath.

MOBILITY.

1. **Mobility by Flagella**, delicate hair-like appendages which according to some investigators are out-growths of the cell membrane, or of the cell wall itself. A greater majority, however, believe them to be out-growths through the cell membrane from the protoplasm. They are called:—

- (a) **Monotrichous**, when situated singly at one pole.
- (b) **Amphitrichous**, when situated singly at each pole.
- (c) **Lophotrichous**, when situated plurally at each pole.
- (d) **Peritrichous**, when scattered around the entire cell. They

move the organism fast or slow in any direction away from its original position when first observed.

They are very difficult to demonstrate as they are very delicate, easily break off and disintegrate. Dark-field illumination and special staining methods are required for their demonstration.

2. **Locomotion by undulating membranes** has been observed in some bacteria.
3. **Amoeboid locomotion** has been found in rare instances.
4. **Brownian, vibratory or molecular movement.** The bacteria vibrate, but do not change their position. The movement is due to transmission by external physical causes.

REPRODUCTION.

Under this head is considered the:—

1. **Active Stage (Vegetative)**, i. e. by **fission or simple cell division**. When conditions such as heat, moisture and nutrition are favorable, together with the absence of the deleterious effects of other bacteria, or their products
 - (a) The cell becomes elongated and the protoplasm aggregates at opposite poles.
 - (b) The cell-wall constricts, usually midway between the protoplasmic aggregations, gradually forming a septum in the interior of the cell.
 - (c) The septum divides the cell into two equal parts.
 - (d) The daughter cells may remain united by the gelatinous envelope for a variable time. Eventually they separate and they themselves subdivide.

This division may take place in one, two or three planes, depending upon the nature of the organism.

Division may be completed in less than 30 minutes.

2. **Resting Stage (Sporulation).** **Spore formation**, is endogenous (Endosporous) or Arthrogeous (Arthrosporous).

The requisites for spore formation were once supposed to be:—

- (a) An exhaustion of nutriment.
- (b) The generation within the medium of toxic material from the accumulation of metabolic products.
- (c) The environment becomes unfavorable, e. g., temperature.

In other words, when conditions became such that the cell could no longer maintain life, the organism turned itself into a spore in order that it might escape annihilation.

This is not altogether correct, as sporulation takes place only when conditions present are most favorable to the well-being of the cell. The temperature at which spores are best formed is constant for each organism, but varies with the different species, aerobes require oxygen for sporulation but anaerobes will not spore in its presence.

Endogenous spore formation. The protoplasm of the cell becomes differentiated and concentrates into a small granule which increases in size, or several granules are formed, which coalesce and grow to form an oval or rarely cylindrical mass. Further contraction takes place, the outer layers become still more differentiated and form a distinct spore membrane. Some authorities maintain that the spore membrane consists of two layers, the exosporium and the endosporium.

The spore is now a clearly defined highly refractile body.

The cell contains but one spore, situated usually in the middle, occasionally at one end (four exceptions have been recorded, e. g. *B. inflatus*). It is of the same diameter, or a little less, as that of the cell itself.

The shape of the parent cell may be unaltered (e. g. *B. anthrax*) or altered (e. g. *B. tetanans*), and this serves for a classification of spore-bearing bacilli: viz:—

- (a) Cell body unaltered in shape.

(b) Cell body altered in shape. The terms applied to each are:—

(1) **Clostridium.** (Spindle shape). Swollen at the center and thin at the poles.

(2) **Cuneate.** (Wedge-shape).

(3) **Clavate.** (Key-hole shaped). Swollen at one pole and unaltered at the other.

(4) **Capitate.** (Drum-stick shaped).

The endospores remain within the cell for a variable time, but are eventually set free by the swelling up and the solution of the cell membrane of the parent by the surrounding liquid or by the rupture of the membrane. The spore now presents the following characteristics:—

(a) Well formed, dense cell membranes, rendering their staining difficult; and when stained, equally difficult to decolorize.

(b) Highly refractile, which differentiates it from vacuoles.

(c) Higher resistance (**spore resistance**) than the parent organism on account of the low water content of plasma, low heat conducting power and the low permeability of the spore membrane to such lethal agents as chemicals, light, heat, desiccation, starvation, time, etc., this resistance varying somewhat with the particular species.

Bacteria grown on media poor in nutrient material are likely to become **asporogenous**; i. e., they become sterile and do not produce spores. This condition may be temporary or permanent.

Arthrospore formation is seen only in the micrococci. One complete element resulting from fission becomes differentiated for this purpose, enlarges, and develops a dense cell wall. This process is probably not real spore formation but a relative increase in resistance. They have never been seen to germinate, nor is their resistance very marked, as they fail at culture after having been exposed to 80° C temperature for 10 minutes.

Spore Germination. When placed under favorable conditions of heat, mois-

ture, nutrition, etc., the spores germinate, usually within 24 to 36 hours and successively undergo the following changes:—

- (1) Swell up slowly and enlarge, through absorption of water.
- (2) Lose their refrangibility (grow dull).
- (3) One of the following processes is observed (a particular process is constant for the same species):—
 - (a) The spore grows out into the new organism without throwing off its membrane.
 - (b) It loses its spore membrane by solution.
 - (c) It loses its spore membrane by rupture.
 - (d) Endo-germination. The spores germinate within the parent body. The germinal rod becomes detached, leaving the empty capsule within the parent.

The rupture may be polar or equatorial.

The polar rupture may take place at one pole only or at both poles.

In the cases where the spore membrane is discarded, the cell membrane of the new bacillus may be formed from:—

- (a) The inner layer of spore membrane, which has been split up into a parietal and visceral layer.
- (b) The outer layers of the cell protoplasm, which has become differentiated for that purpose.

The new organism now increases in size, elongates and takes on vegetative growth.

Formations of Gonidia. In the higher bacteria (filamentous bacteria), as in *Mycobacteriaceae*, a number of specialized cells or spores are formed (short rods or coccoid forms) by multiple segmentation or differentiation, usually at the free tip of the filament, and are termed gonidia (conidia).

They may be termed resting bodies, as they remain dormant for a variable period until favorable conditions are brought about, when they

elongate and produce the vegetative form from which they arose.

Many of these gonidia have been considered as degenerative forms, but this is unlikely as degenerative elements would not produce new vegetative cells. According to A. Coffen Jones, tubercle bacilli produce gonidia.

The resistance of the diphtheria organism to unfavorable conditions would make it likely that the granular segments so often produced are of the nature of gonidia.

BIOLOGICAL CLASSIFICATION.

1. Bacteria are classified according to their **life functions** into:—

(a) **Saprogenic**. (Saprophytes), or putrefactive bacteria, are those that live only on dead organic matter.

(b) **Zymogenic**, or fermentative bacteria, are those which produce soluble ferments or enzymes during the course of their growth. The ferments possess the power of breaking up more highly organized nitrogenous and non-nitrogenous compounds into simple and more diffusible substances. The action of ferments upon non-nitrogenous compounds is called **fermentation**. The action of ferments upon nitrogenous compounds is called **putrefaction**, often producing odorous gases and ptomaines, which are complex alkaloids resembling those found in plants.

The principal bacterial ferments are:—

Proteolytic (Converts proteins into proteose, peptone and further products of hydrolysis).

Diastase (Converts starches into sugar).

Invertase (Converts saccharose into a mixture of dextrose and levulose).

Rennin or coagulating (Coagulates milk independent of the action of acids).

(c) **Pathogenic**, or disease producing bacteria, are those causing

various pathological conditions and producing the diseases known as (**infectious diseases**).

2. Bacteria are classified according to their **food requirements** into:—

(a) **Prototrophic**, (e. g. nitrifying bacteria) are those which require no organic food.

They change albuminoids into skatol, indol, leucin, and these into nitrites and nitrites into nitrates.

(b) **Metatrophic**, (e. g. saprophytes and facultative parasites), are those which require organic food.

The saprophytes are easily cultivated; some will grow in almost pure distilled water and some will grow in pure solutions of carbohydrates.

The facultative parasites need highly organized foods as proteids or other sources of nitrogen and carbon and salts.

(c) **Paratrophic**, (e. g. obligate parasites) are those which require living food. They will not live outside the living body.

3. Bacteria are classified according to their **metabolic products** into:—

(a) **Chromogenic**, or pigment-producing bacteria, are those which produce vivid pigments (yellow, orange, red, violet, fluorescent, etc.,) during the course of their life and growth. The coloring matter is usually an intercellular excrementitious substance; though it occasionally appears to be stored within the body of the organism. They are therefore classified into:—

Chromoparous bacteria, when the pigment is diffused out upon and into the surrounding medium.

Chromophorous bacteria, when the pigment is stored in the cell protoplasm of the organism.

Parachromophorous bacteria, when the pigment is stored in the cell wall of the organism.

Different species of chromogenic bacteria differ in their requirements as to environment for the production of their character-

istic pigments; some need oxygen, light or high temperatures; others favor the opposite conditions.

(b) **Photogenic**, or light-producing bacteria, are those which exhibit phosphorescence when cultivated under suitable conditions.

(c) **Aerogenic**, or gas producing bacteria, are those which produce hydrogen, carbon dioxide and sulphuretted hydrogen, etc.

Toxins. Many bacteria, especially the pathogenic, elaborate or secrete poisonous substances, concerning which little exact knowledge is available, though many appear to be enzymic in their action. They seem to be akin to the venom of serpents and other animals and to certain poisonous principles of plants.

It has been estimated that 1-1000 gm. of tetanus toxin will kill a horse weighing 1,200 pounds. They were first called ptomaines or cadaveric alkaloids, but this term is now applied to poisons which form in decomposing meat, cheese, etc., as a result of chemical change caused by bacteria; they have also been termed **toxalbumins**, as they give all the reactions of albumin. It is probable, however, that a toxalbumin is but a combination of the toxin and the substances derived from the medium of growth.

A certain group of toxins are retained within the organism and are only set free after its death.

Toxins are usually divided into:—

Intracellular (inseparate) are those which are bound up with the protoplasm of the organism.

No means has as yet been devised for their separation or extraction. **Anti-bacterial serum** is used to combat this type.

Extracellular (soluble) are excreted by the organism and are diffused into and held in solution by the surrounding medium.

Anti-toxin serum is used to combat this type.

End-products of metabolism are organic acids (lactic, butyric, propionic, benzoic, formic, acetic, oxalic,

succinic, salicylic, gallic and tannic), alkalies (ammonia), aromatic compounds (indol, phenol), reducing substances (nitrates to nitrites), and gases (sulphuretted hydrogen, carbon dioxide, etc.)

Growth. Certain conditions are necessary to the life and growth of bacteria; any marked change in these conditions will inhibit the growth or destroy them. Water is absolutely essential for their growth.

1. **Influence of atmosphere.** Certain bacteria require oxygen for their growth and death will follow if this is not available. They are termed **obligate aerobes**.

A certain group of bacteria will thrive equally well with or without oxygen. They are termed **facultative anaerobes**.

Certain bacteria live and multiply only when there is complete exclusion of free oxygen. They are termed **obligate anaerobes**.

2. **Influence of heat.** A temperature of from 10° to 40° C is necessary to the life and growth of bacteria. Practically no growth occurs below 5° C., and very little above 40° C. The most favorable temperature for the majority of microorganisms is from 30° C to 37° C. Saprophytes grow between 0° and 30° C., the optimum being 15° to 20° C.

Parasites flourish between 10° and 45° but best at body temperature, 37° C.

The maximum and minimum temperatures at which growth takes place, as well as the optimum, are fairly constant for each bacterium. They may be classified, according to their optimum temperature, into:—

	MIN.	OPT.	MAX.
(a) Psychrophilic (chiefly water organisms)...	0° C.	15° C.	30° C.
(b) Mesophilic (includes pathogenic forms)	15° C.	27° C.	45° C.
(c) Thermophylic bacteria	45° C.	55° C.	70° C.

Each bacterium has its own **Thermal death point**.

The "thermal death point" of an organism is that temperature which causes the death of the vegetative forms when the exposure is continued for a period of 10 minutes.

It is between 50° and 60° C in the most pathogenic, while below the lower limit their growth is only inhibited. An exposure to 250° C. has been made without preventing the organisms future development.

Spores are extremely resistant; some are killed only after an hour's exposure to 115° C.

3. **Influence of light.** Many organisms are indifferent to the presence of light. On the other hand

Daylight frequently inhibits the growth and alters to a greater or lesser extent the biochemical characters of the organisms; e. g., chromogenicity or power of liquefaction. Pathogenic bacteria undergo a progressive loss of virulence when cultivated in the presence of daylight.

Direct sunlight destroys them as does also electric light, but to a less extent. Violets rays are very effective in the destruction of bacteria.

4. **Influence of electricity.** Electrical currents inhibit or destroy the growth of bacteria, not directly, but probably by the products of electrolysis.

Roentgen rays are bactericidal to bacteria in living tissues, but have little effect on cultures.

5. **Influence of movements.** Movements, if slight and of a flowing character, do not seem to affect the growth of bacteria, but violent shaking kills them.

CULTIVATION OF BACTERIA.

Culture Media, Tubing Media, Sterilization.

As it is difficult and sometimes impossible to study the growth of bacteria in their natural habitat, it becomes necessary to isolate individual members of microorganisms, to observe their growth, morphology, phenomena, etc., by their cultivation on artificial nutrient media.

APPARATUS REQUIRED.

Test tubes. Several sizes should be kept in stock. The ordinary tubes in most use are the $\frac{5}{8}$ "x5" Board of Health tubes. Small tubes 5x0.9 cm. for use in inverted position inside tubes containing carbohydrate media as gas collecting tubes.

Florence Flasks of 250, 500 and 1000 cc. capacity will be found very convenient.

Erlenmeyer Flasks, with narrow neck of 75, 100, 150 and 250 cc. capacity.

Petri Dishes or Plates, 1.5 cm. high X 10 cm. diameter put up in bundles of 6, wrapped in paper or cloth, sterilized and put aside for use. They can also be put up in specially prepared metallic boxes.

Pipettes of 1 cc. plain, 1 cc. graduated in 0.1 cc., and .01 cc. capacity; also pipettes of 10 cc. capacity graduated in .1 cc. Each variety should be stored in large test tubes or in special metallic boxes, sterilized and put aside for use.

Capillary pipettes (Pasteur's) are prepared from small bore soft glass tubing, heated and pulled out to a fine capillary tube at one end.

Blood pipettes (Pakes) are made, from 1 cm. bore soft glass tubing, in a manner similar to Pasteurs, except that they are pulled out at both ends. Wright's tubes are similar to Pakes' except that one end is turned at an angle. They are stored in test tubes, sterilized and put aside for use.

Fermentation tubes, used for the collection and analysis of gases liberated from media during the growth of some bacteria. They may be plain or graduated. They are plugged with cotton and sterilized.

Platinum wire, fitted into a glass or aluminum handle, to be used for inoculations.

CULTURE MEDIA.

The greater number of these media are **primarily fluid**, but in order to better study the characteristics of individual organisms, through their colonies many media are therefore

rendered solid by the addition of substances like gelatin or agar in varying proportions. Gelatin is employed for the solidification of those media on which it is intended to cultivate bacteria at room temperature or in the "cold" incubator. Gelatin, in the percentage usually employed, becomes liquid at 25° C.

Agar, in the percentage usually employed, only becomes liquid when exposed to 90° C for a considerable period and again solidifies at 40° C.

Such media is spoken of as a **liquefiable media**. Other media as potato, coagulated blood serum, etc., can not be again liquefied and are therefore spoken of as **solid media**.

Meat Extract forms the basis of several of the nutrient media and is prepared as follows:—

1. Add to 1000 cc. distilled water, in an enameled pot, 500 gms. of finely minced fresh lean meat.
2. Heat gently in a water bath, at a temperature that at no time exceeds 40° C, for 20 minutes; this will dissolve out the soluble proteids, extractives, salts, etc.
3. Raise the temperature of the mixture to boiling and maintain for 10 minutes; this precipitates some of the albumins, haemoglobin, etc., from the solution.
4. Strain through muslin (sterile) or a perforated porcelain funnel, then filter through paper into a sterile flask and when cool make up loss by evaporation to 1000 cc. with distilled water.
5. If not needed at once, sterilize for 20 minutes on 3 consecutive days.

Wyeth's beef-juice, or Liebig's extract of meat, 3 gms. to 1000 cc. of distilled water heated and filtered as above, may be substituted, except where the more highly parasitic bacteria are to be cultivated.

The Reaction of Meat Extract as prepared above is always acid, due to acid phosphates of potassium and sodium, acids of the glycollic series, and acid organic compounds.

Prolonged boiling causes the extract to undergo hydrolytic changes which increase its acidity. It should therefore be boiled for at least 45 minutes, when it will become stable, and the total acidity is to be estimated when the solution is at the boiling point.

The meat extract reacts acid to phenolphthalein, though it may react neutral or alkaline to litmus, due to

- (1) The insensitiveness to some organic acids.
- (2) The formation of dibasic sodium phosphate, formed during the process of neutralization.

STANDARDIZING THE REACTION.

1. Fill a burette with standardized $n/20$ NaOH.
2. Measure out 5 cc. of media and 45 cc. distilled water into a beaker (should be at a temperature of 100° C).
3. Add to the contents of the beaker, 5 drops of a 0.5% (50% alcohol) solution of phenolphthalein.
4. From the burette, run the $n/20$ NaOH solution carefully into the test media, constantly stirring until the end-point is reached, as indicated by a deep-rose color.
5. Read off the amount of NaOH solution required to neutralize the 5 cc. of media.
6. Verify the reaction by another titration.
7. Calculate the amount of standardized normal NaOH it will take to neutralize the remaining 990 cc. of media.

(For all practical purposes it can be estimated as still having 1000 cc. of media and adding the normal NaOH, viz:—if it requires 5 cc. of the $n/20$ NaOH to neutralize 5 cc. of the media, then 50 cc. of the normal NaOH will be required to neutralize the 1000 cc. of media. In other words, move the decimal point one to the right, e. g., burette reading is 5.3 cc., then 53. cc. will neutralize the 1000 cc. of media). The sign + (plus) is prefixed to the media if it is

acid and the sign — (minus) if it is alkaline, e. g., media + 10 indicates that it reacts acid to phenolphthalein and would require the addition of 10 cc. normal NaOH per 100 cc. for neutralization.

8. Titrate again the neutralized media to insure results. In as much as the titration for a last control is often wanted, it may be well to use a deka-normal NaOH for the neutralization of the bulk, so as not to bring the total quantity of media greatly above the original 1000 cc., as might be the case if the N/20 or normal NaOH were used.

Nearly all bacteria have a well marked "optimum reaction" which happily approximates close to + 10, therefore this standard may be used for all media unless otherwise indicated.

The standardizing 1000 cc. of media to + 10 is accomplished by merely subtracting 10 of the NaOH from the initial calculation. This renders the reaction + 10.

FILTRATION OF MEDIA.

Fluid Media are filtered through filter paper folded in the "physiological-filter form so as to accelerate the rate of filtration.

Liquefiable Media are filtered through "paper Chardin," which is a specially made filter paper.

Gelatin if made properly will filter through this paper readily.

Agar, likewise, if properly made will filter readily, but not so rapidly as gelatin.

A special hot-water jacket has been constructed to surround the glass funnel; the temperature of the water in the jacket is maintained at 90° C. and facilitates the filtration. If care is taken the liquefiable media can be filtered through absorbent cotton efficiently.

STOCK MEDIA.

Bouillon. Put 500 cc. double strength meat extract into a litre flask and add 300 cc. distilled water.

Mix 10 gms. peptone and 5 gms. salt into a smooth paste with 200 cc. of distilled water previously heated to 60° C. Add the emulsion to the meat extract and heat in the Arnold for 45 minutes to dissolve the peptone and to render the acidity of the meat extract stable. Estimate the reaction and control the results. Heat again in Arnold for 30 minutes to completely precipitate the phosphates. Filter through paper into flask. Sterilize, or tube and sterilize.

Agar=Agar. (Agar is derived from sea plants along the coast of Japan. It has some of the properties of gelatin, but is less affected by heat).

Weigh a 2 litre double Agate ware boiler and note it. Put 500 cc. double strength meat extract into the boiler. Mix 10 gms. of peptone, 5 gms. of salt into a paste with 150 cc. distilled water. Add the paste and 15 to 20 gms. of Agar (powdered if available) to the meat extract. Heat over flame to 100° C. for 25 minutes (stirring constantly) or more for complete solution of Agar. Weigh the pan and to its contents add enough water to make up the bulk of the medium to 1 litre.

Titrate, control the result, calculate the amount of soda solution required to make the medium + 10 and add it to the medium. Place in the Arnold or over the flame for 20 minutes to complete the precipitation of the phosphates, etc.

Cool the medium to 60° C., add the whipped white of two eggs, place it over the gas burner or in Arnold until the egg-albumin has formed into a firm floating mass. Filter through paper, tube and sterilize.

Gelatin is used for determining the proteolytic ferments of bacteria by its liquefaction. Other distinctions are also met with.

Weigh a 2 litre double Agate boiler and note it. Put 500 cc. double strength meat extract into the boiler. Mix 10 gms. of peptone, 5 gms. of salt into a paste with 150 cc. distilled water. Add the paste and 100 to 150 gms. sheet gelatin (cut into small pieces) to the meat extract. Heat over flame to 100° C. for 10 minutes (stirring constantly till there is complete solution of the gelatine. Weigh the pan and its contents and add enough water to make up the bulk of the medium to 1 litre. Titrate, control the result, calculate the amount soda solution required to make the medium + 10 and add it to the medium. Place in the Arnold or over the flame for 20 minutes to complete the precipitation of the phosphates, etc. Cool the medium to 60° C., add the whipped white of two eggs, place it over the flame or in the Arnold until the egg albumin has formed into a firm floating mass. Filter through paper, tube and sterilize.

Blood Serum. The blood is collected at the slaughter house in sterile glass cylinders and allowed to stand for 15 minutes to form clot to prevent the serum from being stained with haemoglobin. When removed to the laboratory the clot is separated from the sides of the cylinder by a sterile glass rod and placed in the ice chest for 24 hours. The serum is then drawn out with sterile pipettes and placed in sterile test tubes (5 cc. in each). The tubed serum is heated on two successive days. The third day, heat the tubes in a slanting position in a serum inspissator to about 72° C. which coagulates the serum. Place the tubes in the incubator at 37° C. for 48 hours to eliminate the tubes that have been contaminated. Store in a cool place. The serum can be sterilized by the fractional method by exposure in a water bath to a temperature of 56° C. for 30 minutes on each of 6 consecutive days. Store in the fluid condition and coagulate in the inspissator when needed.

Guy's Citrated Blood Agar. A small rabbit is killed by chloroform, nailed out on a board, hair moistened thoroughly with 2% solution of lysol, skin is reflected (with sterile instruments) over the thorax, thorax opened (sterile), pericardium opened (sterile), surface of left ventricle seared with hot iron, the point of a sterile capillary pipette is thrust through the wall of the ventricle, pipette filled with blood by suction, transfer the blood to a small Erlenmeyer flask containing a number of glass beads and 5 cc. concentrated sodium citrate solution, agitate, set aside for 2 hours, with a sterile 10 cc. graduated pipette, transfer 1 cc. citrated blood to a tube of liquefied agar, mix, allow agar to cool in slanting position, place tubes in incubator for 48 hours, after which time store the uncontaminated tubes for future use.

Potato. Cylinders are cut out of a well washed peeled potato. The cylinders are cut obliquely from end to end, forming them into wedges. The fresh potato is strongly acid and in order to approximate + 10 the cylinders are placed in 1% solution of sodium carbonate for 30 minutes. Each wedge is placed in a test tube into which has been previously inserted a piece of absorbent cotton moistened with sterile water, with its base resting upon the cotton. The tubes are then replugged and sterilized in Arnold on each of 3 consecutive days.

The acid of the potato can also be abstracted by placing the wedges in running water for 24 hours.

Dorset's Egg. Sterilize in the autoclave for 20 minutes 1 litre of a .85% solution of sodium chloride and cool to 20° C. Wash 12 fresh eggs with water, then with pure formaline and allow them to dry. Break the eggs into a sterile graduate, noting their total volume. Add the salt solution to the eggs in proportion of 1 to 3. Whip the mixture with an egg-whisk thoroughly and filter through

coarse muslin into a sterile flask. (A few drops of alcoholic solution of basic fuchsin to give a definite pink color, or a few drops of water proof Chinese ink added to the medium at this stage will facilitate the subsequent "fishing of colonies").

Tube and solidify at a slant in the inspissator at 75° C. for one hour. Incubate for 48 hours and eliminate the contaminated tubes. The sterile tubes are capped with rubber caps and stored for future use.

Egg. A number of eggs are broken into a vessel and thoroughly mixed with a little water, tubed and sterilized at a slant in the Arnold on each of 3 consecutive days.

Dunham's Peptone. 10 gms. of peptone and 5 gms. of salt are emulsified with 250 cc. of distilled water previously heated to 60° C. The emulsion is placed in a flask and made up to 1 litre with distilled water. Heat in Arnold for 30 minutes, filter through paper, tube and sterilize in Arnold.

Dextrose Bouillon. Make bouillon in the manner outlined above and add to it 1% of dextrose. Tube and sterilize as for bouillon. This media is generally used in the fermentation tubes. The ordinary glucose will answer as well except that during its sterilization it will deepen greatly in color.

Milk. 1 litre of fresh milk is put into a large separating funnel and heated in the Arnold for 1 hour. Estimate the reaction. (If it is higher than + 20 or lower than + 10 reject it.) Cool to separate the fat. Draw off the fat-free milk into sterile tubes and sterilize in the Arnold for 20 minutes on each of 5 successive days. Incubate for 48 hours and eliminate any contaminated tubes.

Litmus Milk. The milk is prepared as described above, and fat-free is drawn off into a sterile flask. Sufficient sterile litmus solution is added to give it a deep lavender color. Tube and sterilize as above.

SPECIAL MEDIA.

ANAEROBIC CULTURES.

Kitasato's Glucose Formate Bouillon. Dissolve 20 gms. of glucose and 4 gms. of sodium formate in 1 litre bouillon. Tube and sterilize in Arnold.

Weyle's Sulphindigotate Bouillon. Dissolve 20 gms. glucose and 1 gm. of sodium sulphindigotate in 1 litre bouillon. Tube and sterilize in Arnold.

Kitasato's Glucose Formate Gelatine. Dissolve 20 gms. of glucose and 4 gms. of sodium formate in 1 litre of hot gelatin. Filter through paper, tube and sterilize in Arnold.

Weyl's Sulphindigotate Gelatin. Dissolve 20 gms. glucose and 1 gm. of sodium sulphindigotate in 1 litre hot gelatine. Filter through paper, tube and sterilize in Arnold.

Kitasato's Glucose Formate Agar. Dissolve 29 gms. glucose and 4 gms. sodium formate in 1 litre hot agar. Tube and sterilize in Arnold.

Sulphindigotate Agar. Dissolve 20 gms. glucose and 1 gm. sodium sulphindigotate in 1 litre hot agar. Tube and sterilize in Arnold.

All the sulphindigotate media are of a blue color. During the growth of the anaerobes, the media is oxidized and changed in color to a light yellow.

MacConkey's Bile Salt Broth. Emulsify 20 gms. of peptone in 200 cc. distilled water previously warmed to 60° C. Dissolve 5 gms. sodium taurocholate and 5 gms. of glucose in the emulsion. Wash the emulsion into a flask with 800 cc. of distilled water and place in Arnold for 20 minutes at 100° C. Filter through paper and add sterile litmus solution until the medium is of a deep purple color. Tube into gas tubes and sterilize in Arnold for 20 minutes on 3 consecutive days.

FOR THE STUDY OF THE ORGANISM'S CHEMICAL COMPOSITION.

Uschinsky's Asparagine. Dissolve 3.4 gms. asparagine, 10 gms. ammonium lactate, 5 gms. sodium chloride, 0.2

gms. magnesium sulphate, 0.1 gm. calcium chloride and 1 gm. acid potassium phosphate, in 1 litre of distilled water. Add 40 cc. glycerine, tube and sterilize in Arnold.

This media can be made up into gelatine or agar.

Uschinsky's Proteid Free Broth. Dissolve 0.1 gm. calcium chloride, 0.2 gms. magnesium sulphate, 2 gms. acid potassium phosphate, 3 gms. potassium aspartate, 5 gms. sodium chloride and 6 gms. ammonium lactate, in 1 litre of distilled water. Add 30 cc. glycerine, tube and sterilize.

FOR THE STUDY OF THE ORGANISM'S BIO-CHEMICAL REACTION.

Dunham's Inosite-free Bouillon. Inoculate 1 litre of bouillon with the *B. lactis aerogenes* and incubate for 48 hours. Heat in Arnold for 20 minutes. Estimate the reaction and make it + 10. Inoculate with the *B. coli communis* and incubate for 48 hours. Heat in Arnold for 20 minutes.

Fill 2 fermentation tubes, tint with litmus solution and sterilize; inoculate with the *B. lactis aerogenes*. If no acid or gas is formed the medium is sugar free; but if acid or gas is present, again make the bouillon to + 10 reinoculate with either of the above bacteria and incubate; make another test. Repeat above procedure till neither acid or gas appears.

Stand the medium in a cool place to allow the growth to sediment. Filter the top medium through paper till clear. Tube and sterilize in the Arnold.

Nitrate Bouillon. Dissolve 5 gms. of potassium nitrate in 1 litre bouillon. Tube and sterilize in Arnold.

Litmus Bouillon. Add enough sterile litmus solution to 1 litre of bouillon, to give it a dark lavender color. Tube and sterilize in Arnold, (+ 10 media will usually react faintly alkaline or occasionally neutral to litmus).

Iron Bouillon. Dissolve 1 gm. of ferric tartrate in 1 litre bouillon. Tube and sterilize in Arnold.

Lead Bouillon. Dissolve 1 gm. of lead acetate in 1 litre bouillon. Tube and sterilize in Arnold.

Pake's Nitrate Peptone. Emulsify 10 gms. peptone with 200 cc. ammonia—free distilled water previously heated to 60° C. Wash emulsion into a flask and make up to 1 litre with ammonia-free distilled water. Heat in Arnold for 20 minutes. Dissolve 1 gm. of sodium nitrate in the above. Filter through paper, tube and sterilize.

Rosalic Acid Peptone. Make a .5%, 80% alcoholic, solution, of rosalic acid (corallin) for a stock solution. Add to 100 cc. Dunham's peptone, 2 cc. of the corallin stock solution. Heat in Arnold for 30 minutes. Filter through paper, tube and sterilize.

Pakes' Iron Peptone. Emulsify 30 gms. of peptone with 200 cc. tap water (heated to about 60° C.) Wash it into a flask with 800 cc. of tap water. Dissolve in it 5 gms. of salt and 3 gms. of sodium phosphate. Heat in Arnold for 30 minutes. Filter and tube. Add to each tube 0.1 cc. of a 2% neutral solution of ferric tartrate. Sterilize.

Lead Peptone. Prepare as for iron peptone except to substitute 0.1 cc. of a 1% neutral aqueous solution of lead acetate for the ferric tartrate.

Capaldi-Proskauer No. 1. Dissolve 2 gms. sodium chloride, 0.1 gm. magnesium sulphate, 0.2 gms. calcium chloride and 2 gms. monopotassium phosphate in 1 litre of distilled water. Add to the mixture, 2 gms. of asparagin and 2 gms. of mannite. Take 25 cc. of mixture and titrate it against n/10 sodium hydrate using litmus as an indicator. Calculate amount of sodium hydrate necessary to make the solution neutral to litmus and add it. Filter and add to it 5% of neutral litmus solution. Tube and sterilize in Arnold.

Capaldi-Proskauer No. 2. Dissolve 20 gms. of peptone and 1 gm. of man-

nite to 1 litre of distilled water. Neutralize as in No. 1, filter and add litmus solution as above. Tube and sterilize in Arnold.

Glucose Gelatine. Dissolve 20 gms. of glucose in 1 litre of hot gelatin, filter through paper, tube and sterilize in Arnold.

Glucose Agar. Dissolve 20 gms. of glucose in 1 litre of hot agar, filter, tube and sterilize in Arnold.

Urine Gelatine. Fresh urine with a sp. gr., of 1010 (if above 1010, it is diluted with sterile water until that sp. gr., is reached) is collected in a sterile flask, heated to the boiling point and the reaction estimated and noted. 10% of gelatine is added and the mixture heated in the Arnold for 1 hour. Estimate the reaction to that of the original urine. Cool to 60° C. and clear with egg. Filter through paper, tube and sterilize in Arnold.

Heller's Urine Gelatin. Same as above with addition of 1% of peptone and 0.5% of salt.

Urine Agar. To fresh urine with a specific gravity of 1010 is added 1.5 to 2% of powdered agar. Heat in the Arnold for 1 and ½ hours. Cool to 60° C. and clear with eggs. Filter, tube and sterilize in Arnold.

Hiss' Serum Dextrose. Blood is collected and allowed to clot. It is then expressed in a graduated cylinder and for every 100 cc. of serum 300 cc. of distilled water is added. Heat in the Arnold for 30 minutes. Filter if turbid. (If not needed at once it can be sterilized for 3 days and stored).

Dissolve 10 gms. of dextrose in 1 litre of the above serum. Filter through paper and add 50 cc. of the "neutral litmus solution." Tube and sterilize in Arnold.

FOR THE STUDY OF CHROMOGENIC ORGANISMS.

Eisenberg's Milk Rice. Mix 70 cc. bouillon and 210 cc. milk. In a mortar rub up 100 gms. of rice powder and the mixture into a paste. Spread the paste out in 0.5 cm. thick layer over the bottom of

petri-dishes. With the lids removed, heat over a water bath at 100° C. until the mixture solidifies.

Replace the lids and sterilize in the Arnold.

FOR THE STUDY OF PHOSPHORESCENT AND PHOTOGENIC ORGANISMS.

Fish Bouillon. Dissolve 26.5 gms. sodium chloride, 0.75 gm. potassium chloride and 3.25 gms. magnesium chloride in 500 cc. distilled water. Add the solution to 500 gms. of herring, cod or mackerel (in an enameled pot) and heat over a water bath, gently at 40° C. for 20 minutes, then rapidly raise to 100° C., maintaining it at this temperature for 10 minutes. Strain through muslin. Emulsify 5 gms. of peptone in 200 cc. of fish water, then mix it thoroughly with the rest. Heat in the Arnold for 20 minutes. Filter through paper and when cold make up to 1 litre with distilled water. Tube and sterilize in Arnold. If it is to be used as a basis for gelatin or agar it should be made up double strength. The gelatine or agar is then prepared in the ordinary manner.

FOR THE STUDY OF EARTH BACTERIA.

Lipman and Brown's Earthy Salts Agar. (Enumeration of soil organism). Emulsify 20 gms. agar with 200 cc. distilled water and wash it by means of 400 cc. of distilled water into a double boiler. Add to it an emulsion made with .5 gm. of peptone and 50 cc. of distilled water and heat at 100° C. for 20 minutes. Add to the mixture, 10 gms. of dextrose, 0.5 gm. potassium phosphate, 0.2 gm. magnesium sulphate and 0.06 gm. of potassium nitrate. Adjust the weight of the mass to the calculated figure for 1 litre (1025 gms.) by adding distilled water at 100° C. Titrate and adjust the reaction to + 5. Cool to 60° C., clear with egg, filter, tube and sterilize, in Arnold.

Beyrinck's No. 1. (Cultivation of nitrogen fixing organisms). Dissolve 1 gm. potassium hydrogen phos-

phate, 0.2 gm. magnesium sulphate and 0.02 gm. sodium chloride in 1 litre of water. Add 1 cc. of a 1% solution of manganese sulphate. Add 20 gms. dextrose and heat in Arnold for 20 minutes. Filter, tube and sterilize.

Beyrinck's No. 2. (For growth of *Azobacter*). It is the same as No. 1, except that mannite is substituted for dextrose.

Winogradsky's for Nitric Organisms. Dissolve 1 gm. potassium phosphate, 0.5 gm. magnesium sulphate, 0.01 gm. calcium chloride and 2 gms. sodium chloride in 1 litre of distilled water. Fill into flasks in quantities of 20 cc. and add to each a small amount of freshly washed magnesium carbonate. Sterilize in Arnold as usual. Add to each flask 2 cc. of a sterile 2% solution of Ammonium sulphate. Incubate and eliminate any flask not sterile.

Winogradsky's for Nitrous Organisms. Dissolve 1 gm. ammonium sulphate and 1 gm. of potassium sulphate in 1 litre of distilled water. Add 5 to 10 gms. basic magnesium carbonate previously sterilized by boiling. Fill into flasks, etc., as for the nitric organisms.

FOR THE STUDY OF WATER ORGANISMS.

Hesse and Heyden Naehrstoff Agar. (For enumeration of organisms) Emulsify 12.5 gms. of agar in 250 cc. of distilled water. Wash the emulsion into a double boiler with 250 cc. of distilled water. Heat in water bath till agar is dissolved and add to it an emulsion made from 7.5 gms. Naehrstoff-Heyden (albumose) with 200 cc. cold distilled water.

Adjust weight of mass (1020 gms.) to 1 litre by adding distilled water at 100° C. Clear with eggs. Tube and sterilize in Arnold.

FOR THE STUDY OF PLANT ORGANISMS.

Haricot Bouillon, (For bacteria from tubercles of legumens). Add to 1 litre of distilled water, 250 gms. of haricot beans, 10 gms. sodium

chloride and 1 cc. of a 1% solution of sodium bicarbonate. Heat in Arnold for 30 minutes. Filter and add 20 gms. saccharose. Tube and sterilize.

Haricot Agar. In the usual way add 15 gms. of agar to haricot bouillon, adjust the weight and reaction, cool to 60° C., clear with eggs. Filter, add the 20 gms. saccharose, tube and sterilize.

Hay Infusion. Macerate 10 gms. of chopped hay with 1 litre of distilled water that has been heated to 70° C. in a flask; close flask with rubber stopper and place in a water bath at 60° for 3 hours. Replace the stopper with a cotton plug and heat in Arnold for 1 hour. Filter through paper, tube and sterilize.

Beets, Carrots, Turnips and Parsnips are prepared in the manner described for potato.

FOR STUDY COLI-TYPHOID GROUP OF ORGANISMS.

Carbolized Bouillon. Dissolve 1 gm. of carbolic acid in 1 litre of bouillon. (2.5 to 5 gms. are also used.) Tube and sterilize in Arnold.

Carbolized Gelatin. Dissolve 5 gms. of carbolic acid in 1 litre of hot nutrient gelatin. Tube and sterilize in Arnold.

Carbolized Agar. Dissolve 1 gm. of carbolic acid in 1 litre of hot nutrient agar. Tube and sterilize in Arnold.

Parietti's Bouillon. Mix 4 cc. of pure hydrochloric acid with 100 cc. of a 5% carbolic acid solution and allow to stand for a few days. Prepare several nutrient bouillon tubes, each containing 10 cc., sterilize, add to each the above solution by means of a sterile pipette in quantities of 0.1, 0.2 and 0.3 cc. Incubate for 48 hours to eliminate the contaminated tubes.

Litmus Gelatin. Add to nutrient gelatin enough sterile neutral litmus to give it a deep lavender color. Tube and sterilize in Arnold.

Litmus Lactose Bouillon. Emulsify 4 gms. of peptone with 200 cc. of meat extract at 60° C. Mix 2 gms. of salt and 20 gms. of lactose with the emulsion.

Add to the mixture 200 cc. of meat extract and 600 cc. of distilled water and heat in Arnold for 30 minutes. Neutralize carefully to litmus paper. Heat in Arnold for 20 minutes. Filter through paper and add sterile litmus solution to color medium a deep purple. Tube and sterilize in Arnold.

Wurtz's Litmus Lactose Gelatin. Render 1 litre nutrient gelatin — 5. Heat in Arnold for 20 minutes. Clear with egg. Dissolve 20 gms. lactose in the medium. Filter and add enough litmus solution to color medium a pale lavender. Tube and sterilize in Arnold.

Wurtz's Litmus Lactose Agar. Render 1 litre nutrient agar — 5. Heat in Arnold for 20 minutes. Cool to 60° C. and clear with egg. Dissolve 20 gms. lactose solution to color medium. Filter and add enough litmus solution to color medium a pale lavender. Tube and sterilize in Arnold.

MacConkey's Bile Salt Agar. Emulsify 15 gms. powdered agar with 200 cc. tap water at 60° C. Mix the emulsions. Dissolve 5 gms. sodium taurocholate in 300 cc. tap water and with it wash the emulsions into a double boiler. Heat in water bath or Arnold for 20 minutes. Adjust the weight of the medium mass for 1 litre (1040 gms.) Cool to 60° C. and clear with eggs. Filter and add 10 gms. lactose. Tube and sterilize in Arnold.

Fawcus' Bile Salt Agar. Emulsify 20 gms. of agar with 100 cc. of distilled water. Wash the emulsion into a double boiler with 500 cc. of distilled water. Heat until the agar is dissolved. Cool to 60° C. and clear with eggs. Filter and add 5 gms. of sodium taurocholate, 20 gms. of peptone and 5 gms. of lactose. Adjust reaction to + 15. Filter, add 20 cc. of a 1% aqueous solution of picric acid. Tube and sterilize in Arnold.

Glycerine Potato Bouillon. Grate 1 kilo of potatoes previously well washed and peeled. Weigh and add distilled water in proportion of 1 cc. for every gm. of potato. Place in

ice chest for 12 hours. Strain and filter through paper. Note amount. Add an equal quantity of distilled water. Heat in Arnold for 60 minutes. Add 4% glycerine. Mix and filter. Tube and sterilize.

Elsner's Potato Gelatine. Grate 1 kilo of potatoes previously well washed and peeled. Weigh and add distilled water in proportion of 1 cc. for every gm. of potato. Place in ice chest for 12 hours. Strain and filter through paper. Note the amount. Add 15% of gelatin and place in Arnold till dissolved. Estimate the reaction and adjust it to + 25. Cool to 60° C. and clear with eggs. Add 1% powdered potassium iodide. Filter through paper, tube and sterilize in Arnold.

Braum's Fuchsin Agar. Prepare a fuchsin solution as follows:— Dissolve 3 gms. basic fuchsin in 60 cc. absolute alcohol. Put aside for 24 hours, then centrifugalize thoroughly and decant the top fluid and place in a well-stoppered bottle.

Dissolve 10 gms. lactose in 1 litre nutrient agar. Adjust the reaction to — 5. Filter and thoroughly mix with 5 cc. of fuchsin solution. Add to the mixture 25 cc. of a freshly prepared 10% aqueous solution sodium sulphite. Tube and sterilize. Store in a dark place.

FOR THE STUDY OF MILK ORGANISMS.

Litmus Whey. Curdle fresh milk by adding rennet and warming to 60° C. Filter off the whey and neutralize to litmus by adding a 40% solution of citric acid. Heat in Arnold for one hour to coagulate all the proteids. If the whey is cloudy, put it aside in ice-chest for 48 hours, decant and filter into a sterile flask. Add litmus solution till the whey is of a deep purple red color. Tube and sterilize.

Petruschky's Litmus Whey. Add 1.5 cc. of hydrochloric or glacial acetic acid to 1 litre fresh milk. Filter off the casein and neutralize to litmus by adding normal sodium hydrate solution. Boil and neutralize to litmus by adding n/10 sodium hydrate solution. Filter and add

litmus solution till the whey is of a deep purple color. Tube and sterilize.

Whey Gelatin. Curdle fresh milk by adding rennet and warming to 60° C. Filter and estimate the reaction. Add 10% gelatine and place in Arnold till dissolved. Weigh and estimate the reaction of the mass. Restore the mass to the original reaction of the whey by sodium hydrate solution. Cool to 60° C. and clear with eggs. Filter, tube and sterilize in Arnold.

Litmus Whey Agar or Gelatin. Add 1.5 cc. of hydrochloric or glacial acetic acid to 1 litre fresh milk and boil for 5 minutes. Filter and render whey faintly alkiline to litmus. Prepare an emulsion from a few cc. of the whey and 10 gms. of peptone. Add the emulsion to the whey. Mix in 50 gms. gelatin (or 15 gms. agar) and heat in the Arnold till dissolved. Clear with eggs, filter and adjust the weight of the medium mass for 1 litre. Add 15 gms. dextrose. Color with sterile litmus solution. Tube and sterilize in Arnold on each of 5 consecutive days.

Gelatin Agar. Emulsify 5 gms. powdered agar with 100 cc. of distilled water. Add to it 400 cc. of double strength meat extract and 100 gms. of gelatin. Heat in the Arnold till dissolved. Emulsify 10 gms. of peptone and 5 gms. salt with 100 cc. double strength meat extract heated to 60° C. and add it to the medium mixture. Heat in the Arnold for 15 minutes, adjust the weight of the medium mass for 1 litre by adding distilled water at 100° C., estimate the reaction and adjust it to + 10; heat in Arnold for 20 minutes, cool to 60° C., clear with eggs, filter through paper, tube and sterilize in Arnold. (This medium will allow incubation at a temperature above 22 C. If incubation at 30° C. is to be employed, use 10% of gelatin and 0.5% of agar in the medium. If incubation at 37° C. is used, make the medium with 12% gelatin and 0.75% agar. Avoid the addition of too much agar, as the

liquefying ferment may be retarded or absent).

FOR THE STUDY OF DIPLOCOCCUS PNEUMONIA.

Washbourn's Blood Agar. Incubate agar slants for 48 hours to evaporate off some of the water of condensation; under aseptic conditions open the thorax of a small rabbit and with sterile pipettes deliver from the heart a small quantity of blood over the surface of each of the agar slants; allow the blood to coagulate in a slanted position; incubate the blood agar for 48 hours and eliminate any contaminated tube.

FOR THE STUDY OF DIPLOCOCCUS MENINGITIDIS INTRACELLULARIS.

Wassermann Ascitic Agar. Add to 210 cc. of distilled water 90 cc. of ascitic fluid and 6 gms. of nutrose. Heat over a flame, with constant shaking, until the fluid boils and the nutrose is dissolved. Add the mixture to 600 cc. of melted nutrient agar, heat in the Arnold for 30 minutes, filter, tube and sterilize.

FOR THE STUDY OF GONOCOCCUS.

Lipschuetz's Egg Albumin Broth. In a flask containing some sterile glass beads place 4 gms. of powdered egg albumin and 200 cc. of distilled water previously warmed to 37° C. Dissolve the egg-albumin by shaking. Add 40cc. of n/10 NaOH. Allow to stand for 30 minutes with frequent shaking. Filter, sterilize by boiling two or three times at intervals of two hours; add 600 cc. nutrient bouillon, fill in quantities of 50 cc. into small flasks, incubate for 48 hours and eliminate any contaminated flask.

Egg Albumin Agar. This is prepared in the same manner as the above except that nutrient agar is substituted for the 600 cc. of nutrient bouillon.

Serum Bouillon. Under aseptic precautions, hydrocele, pleuritic or ascitic fluid is collected in sterile flasks; add to it twice its bulk of sterile nutrient bouillon; if necessary filter; tube, sterilize in water

bath at 56° C. for 30 minutes on each of 5 consecutive days, incubate for 48 hours and eliminate any contaminated tubes.

Wertheimer's Serum Agar. Prepare nutrient agar using 2% of agar, 2% peptone, 5% salt and q. s. meat extract. Adjust the reaction to + 10, filter, tube in quantities of 5 cc. and sterilize. After last sterilization cool to 42° C. and add 5 cc. sterile blood serum from human placenta to each tube, slope the tubes; incubate, when solid, for 48 hours and eliminate any contaminated tubes.

Heiman's Serum Agar. Prepare nutrient agar using 2% of agar, 1.5% peptone, 0.5% salt and q. s. meat extract. Adjust the reaction to + 10, filter, tube in quantities of cc. and sterilize. After the last sterilization cool to 42° C. and add 3 cc. sterile hydrocele fluid, ascitic fluid or pleuritic effusion, to each tube; slope the tubes; incubate, when solid, for 48 hours and eliminate any contaminated tubes.

FOR THE STUDY OF B. DIPHTHERIA.

Loeffler's Blood Serum. Prepare nutrient bouillon using veal meat extract instead of beef. Add to the bouillon 1% of glucose and when dissolved add 300 cc. of clear blood serum to every 100 cc. of bouillon. Fill into a sterile tube and complete as for blood serum.

Councilman and Mallory's Blood Serum. Collect blood in slaughterhouse, coagulate, remove the serum and tube (avoid air bubbles). Heat the tubes at a slant in the hot air sterilizer at 90° C. till coagulated ($\frac{1}{2}$ hour), then sterilize in Arnold for 20 minutes on each of 3 successive days.

FOR THE STUDY OF B. TUBERCULOSIS.

Glycerine Bouillon. Add 60 cc. of glycerine to 1 litre of nutrient bouillon. Tube and sterilize in Arnold.

Glycerine Agar. Add 60 cc. of glycerine to 1 litre of nutrient agar. Tube and sterilize in Arnold.

Glycerine Blood Serum. Add 5% of

glycerine to blood serum before tubing, then proceed as described under Blood Serum.

Glycerinated Potato. Prepare the ordinary potato slants and soak them in 25% solution of glycerine for 15 minutes. Moisten the cotton pads at the bottom of the tube with a 25% solution of glycerine. Tube and sterilize in Arnold for 20 minutes on each of 5 consecutive days.

NEUTRAL LITMUS SOLUTION.

Place 50 gm. of litmus in 300 cc. 95% alcohol and put it aside until alcohol acquires a green color (completed in about 7 days with daily shaking). Decant off the green alcohol and again treat with 300 cc. 95% alcohol and repeat shaking.

Repeat process until on adding fresh alcohol, the fluid only becomes tinged violet.

Pour off alcohol, leaving litmus as dry as possible, connect up bottle to an air pump and evaporate off the last trace of alcohol.

Transfer the dry litmus to a liter flask, measure in 600 cc. distilled water and allow to remain in contact for 24 hours, with frequent shaking.

Filter the solution and add to it one or two drops of pure sulphuric acid until litmus solution is distinctly wine-red in color.

Add excess of pure solid baryta and allow to stand until the reaction is again alkaline.

Filter.

Bubble CO_2 through the solution until reaction is acid.

Sterilize at 100 C. for 30 minutes for 3 days. This also drives off CO_2 , leaving the solution neutral.

TUBING OF NUTRIENT MEDIA.

After filtration the media is placed in flasks or tubes. The flasks and tubes must first have been washed, dried and their mouths firmly closed with a cotton plug about 1 and $\frac{1}{2}$ inches in length (allowing about $\frac{1}{2}$ inch or more to extend beyond the mouth). The flasks and tubes are then placed

in the hot-air oven for about one hour at 150° C. which bakes and molds the plugs and sterilizes the apparatus.

The liquefiable media (liquefied) and the liquid media may be tubed by means of a pipette attached to a funnel by a short rubber tube on which is fitted a pinch-cock to regulate the flow of media into the tube. The tube should contain about 1 and $\frac{1}{2}$ inches or 10 cc. of media.

A special apparatus for tubing media has been constructed that allows the media to be measured into each tube. Fluid media containing carbohydrates may be filled into Smith's fermentation tubes or into the Durham gas tubes; the latter tubes are ordinary media tubes, having smaller tubes inverted inside them. When first filled the small tubes will float on the surface, but after sterilization all the contained air will be replaced by media.

The media must now be sterilized in the Arnold for 3 consecutive days.

STERILIZATION.

Dry Heat produced by means of a "hot-air oven" heated by a gas burner. Hot air at 150° C. will destroy all bacteria and their spores in about 30 minutes. An exposure at about 180° C. for a few minutes only will do the same. This method of sterilization can be used with glass, metal or small bulk fabric only. Large masses of fabric are not readily sterilized by dry heat on account of its poor penetrative power.

MOIST HEAT.

Fractional Sterilization. A water bath with a temperature of 56° C., if maintained for 30 minutes, will destroy vegetative bacteria. It will, however, have no effect on spores.

It is used for sterilizing albuminous fluid media that would coagulate at a higher temperature.

Method. The water bath is heated by a Bunsen flame to 56° C. If the bath is not controlled by a thermo regulator it must be watched carefully. The material to be sterilized

is now placed in the bath so that it will be at least 2 cm. below the level of the water. The temperature of the bath will probably fall somewhat, but will again in a few minutes rise to 56°C . The material is removed and subjected to the rapid cooling effect of running water. The vegetative forms are killed and it is now put for 24 hours in a cool, dark place, at the end of which time some of the spores will have germinated and assumed the vegetative form, these are killed by a similar exposure to 56°C . on the second, third, fourth, fifth and sixth day successively.

The water bath at a temperature of 60°C . for 60 minutes is used in the sterilization of "bacterins," or so-called "vaccines."

One exposure is all that is necessary for reasons to be explained later (see "preparation of bacterins").

Water bath at 100°C . (Water sterilization) destroys vegetative bacteria almost instantly. Spores are destroyed in from 5 to 15 minutes. It is used for metal instruments, rubber stoppers, rubber and glass tubing, etc.

STEAM.

Steam at 100°C . will destroy vegetative bacteria in from 15 to 20 minutes and the spores in from 1 to 2 hours. The various culture media are sterilized by this method.

Koch's Steam Chest was constructed for this form of sterilization. It is a tall, cylindrical vessel divided by a perforated diaphragm into an upper steam chamber and a lower water chamber. The chest is heated by gas-burners.

Arnold's Steam Sterilizer is a modification of Koch's chest. It is very efficient and much used.

Method:

When live steam issues steadily from the sterilizer, the material to be sterilized is placed within the steam compartment and allowed to remain for 20 minutes, if the media is liquid, and for 30 minutes if the media is liquefiable or solid.

This will kill all vegetative bacteria. During the hours of cooling the spores will germinate and can then be destroyed by repeating the process in 24 hours. At the end of another 24 hours the media is subjected to another sterilization.

The method is spoken of as a **discontinuous** or **intermittent sterilization**.

Continuous Sterilization. An exposure to steam at 100° C. for 1 to 2 hours is sometimes practiced, but is not to be recommended.

Superheated Steam. **Chamberland's Autoclave** consists of a metallic cylinder fitted with a movable lid which seals the cylinder by means of bolts. It also has a manometer, vent cock and safety valve. It permits the heating of steam, under pressure to 115° C., and will destroy both vegetative bacteria and spores within 15 minutes. If the pressure is increased so as to raise the temperature to 120° C., the vegetative bacteria and spores will be killed in 10 minutes.

Although it is a short, effective method of sterilization and was formerly employed to a great extent for media, on account of hydrolytic changes in media subjected to high temperatures, which renders it unfit for the cultivation of the more delicate organisms, its use has been restricted to disinfecting old cultures, contaminated articles, etc.

FILTERS FOR STERILIZATION OF AIR AND LIQUIDS.

Cotton wool is used in the laboratory for sterilizing air or gases. It is put as a loose plug in a glass tube or a modified tube (air filter) and sterilized in the hot air oven. If the cotton plug is prevented from becoming moist (from air or liquids) it will prevent organisms from entering.

Porcelain Filters are used for sterilizing liquids.

The liquids are passed through a cylindrical vessel, closed at one end like a test tube, made of either porous "biscuit" porcelain, hard-burnt and unglazed (Chamberland) or of Kieselguhr, a fine diatoma-

ceous earth (Berkfield) and are called "candle" or "bougies." In passing the liquids through these candles, the bacteria are retained in the pores of the filter which renders the liquid germ-free.

BACTERIAL CULTIVATION.

Identification of Bacteria. Culture Characteristics. Staining.

Aerobic Bacteria.

1. **Tube Cultures**, accomplished by means of a straight or a looped end platinum wire fastened to the end of a glass or aluminum rod. The procedure is as follows:
 - (a) Sterilize the wire by heating in a flame.
 - (b) Remove cotton plug from tube and hold the end of plug that has not been within the tube between the fingers.
 - (c) Touch wire to the material to be transferred.
 - (d) Stroke or smear (stroke, or "streak" culture, is made by drawing the wire as lightly as possible along the center of the surface of the medium. "Smear" culture is made by rubbing the loop all over the surface of the medium) the contaminated straight wire over the surface of the slanted media; or if the media is not slanted, "stab" culture the solidified media with the wire. This is also employed for the so-called "shake cultures."
 - (e) Replace the cotton plug.
 - (f) Sterilize the wire in the flame. Label for identification and with the date of inoculation.
 - (g) Place all inoculated tubes, except that containing gelatin, in the incubator.
2. **Plate Cultures**. Petri dishes are used. These are two shallow glass dishes, so made that one will cover the other. These cultures are made in order that the appearances of the separate colonies may be studied. Having first sterilized the Petri dishes in a hot air oven, the procedure for plating the culture is as follows:

- (a) Several tubes of agar or gelatin are melted, then cooled to a temperature not destructive to the bacteria; 42° C. for agar and lower for the gelatin. A water bath with a constant temperature of about 43° C. is very convenient.
- (b) Place 3 sterile Petri dishes in a row and number them 1, 2 and 3.
- (c) Looped-end wire is sterilized over flame.
- (d) A loopful of culture is then shaken in the tube of melted media. This tube is marked No. 1, or first dilution. Shake with an even circular movement so as to diffuse the inoculum throughout the medium.
- (e) Sterilize the loop and transfer 2 loopfuls of No. 1 to another tube of melted media. This is marked No. 2, or second dilution. Mix as before.
- (f) In like manner transfer 3 loopfuls of No. 2 to another tube of melted media and this is marked No. 3, or third dilution. Mix as before.
- (g) Sterilize the wire.
- (h) Flame the plug of tube No. 1, remove it, flame the lips of the tube, raise the cover of Petri dish No. 1 and pour the inoculated liquefied medium into it so as to form a thin layer over the bottom of the plate.
No. 2 and 3 are poured in a similar manner.
Place agar dishes in the incubator. The gelatin dishes are to remain at room temperature.

The dilutions are made in order that the colonies may be thinned out, thus allowing their accurate study and sometimes their separate recovery, when under various conditions there may be more than one kind of bacterial colonies present.

In the pouring of the plates, No. 1 (1st dilution) rarely gives a plate of any value, therefore it is replaced by a tube of bouillon or salt solution; the plate (No. 1) is not poured.

When the main object of the dilutions is to obtain subcultivations from a number of individual bacteria, "**surface plates**" are prepared.

Method. Liquefy three tubes of liquefiable media and pour each tube into a separate Petri dish and allow to solidify. When cold place a drop of the inoculum on the surface of the media close to one side of the plate and with a platinum wire, glass rod or aluminum wire (bend about 4 cm. of one end at a right angle, sterilized) smear the drop over the surface with the short arm of the spreader (holding the plate vertical). Rub the infected spreader over the surface of No. 2 plate then over No. 3 plate. Sterilize the spreader, label and incubate the the plates.

ANAEROBIC BACTERIA.

Anaerobic cultures are made by growing the organisms, by means of culture tubes or plates, in the absence of oxygen. This is accomplished by:—Exclusion of the air from the cultivation; exhaustion of the air from the vessel containing the cultivation by an air pump; displacement of air by an indifferent gas, e. g. hydrogen; absorption of oxygen by means of pyrogallic acid rendered alkaline with caustic soda (nitrogen atmosphere); a combine of two or more of the above.

METHODS.

Hesse's. A deep stab in agar or gelatine is made with the needle containing the organism, and the tube is then nearly filled with melted sterile media, or a layer of sterilized oil is poured upon the surface 1 to 2 cm. deep.

Another method, when dealing with pure cultivation is to make a plate of agar or gelatin, inoculate one spot of the surface and place over the spot a sterile cover slip or piece of mica, well pressed down to exclude air bubbles.

Roux's. Aspirate inoculated media into capillary pipettes and seal each end of pipette in a blow flame.

Another method, sometimes spoken of as the "biological method," is to make a deep stab into gelatin or agar and then pour a layer of a broth cultivation of a vigorous aerobe over it.

Buchner's. An inoculated culture tube is placed within a larger tube, the lower part of which contains an alkaline solution of pyrogalllic acid. The tube is closed with a rubber stopper. Use 1 gm. of pyrogalllic acid for every 100 cc. of air capacity of the larger tube.

Wright's. Make a tube cultivation, cut off the projecting part of cotton plug, push the plug into the tube (2 to 3 cm. distance); with a pipette run about 1 cc. of a 10% solution of pyrogalllic acid onto the plug; with another pipette an equal amount of soda and close the tube quickly with a rubber stopper.

Exhaustion of Air. Make a tube cultivation, replace the cotton plug with a perforated rubber stopper, fit in a glass tube bent at a right angle with a construction of about 3 cm. above the stopper, connect glass tube with a water or air pump (interposing a wash-bottle containing sulphuric acid), exhaust the air and seal the glass tube at the construction, using a blow-pipe flame, before disconnecting the pump.

Novy's. Place cultivations inside Novy's jar, connect up delivery tube with hydrogen apparatus, attach rubber tubing to exit tube, collect sample of issuing gas (over water) for testing; when air is completely displaced turn the stopper to close entry and exit tubes and disconnect the gas apparatus.

Bulloch's. Place cultivations in a glass dish resting in the center of a glass slab; put pyrogalllic acid at one side of the dish; put sodium hydroxide near the pyrogalllic acid; smear flange of Bulloch's jar with resin ointment; smear stop-cocks with resin ointment; connect short tube with gas supply; open both stop-cocks; connect a piece of glass tubing by means of a piece of rub-

ber tubing (with a screw-clamp) to the long tube; collect issuing gas and test; when air is displaced shut off stop-cock of entry; shut off stop cock of exit, screw down clamp; remove glass tube from rubber connection; connect up short tube to air pump; open stop-cock of short tube; aspirate small quantity of gas; shut off stop cock; disconnect air pump; fill 10 cc. bulb pipette with water and insert it into rubber tubing on long tube as far as screw clamp; open screw clamp; run in water till stopped by internal pressure and shut off stop cock. Incubate.

Botkins. Place a leader cross in a glass dish (20 cm. in diameter, 8 cm. deep) put tube cultivation in a glass jar or plate cultivations in a wire frame resting them on the cross, adjust U-shaped pieces of glass tubing in a vertical position on opposite sides of the dish, place a bell jar over the cultures enclosing one arm of each U-tube (resting it on the cross), fill the dish with glycerine or mercury to a depth of about 5 cm. and connect one U-tube with a gas apparatus.

IDENTIFICATION OF BACTERIA.

In order to identify an organism after isolation it must be studied as to cultural characters, morphology, chemical products of growth, biology and pathogenicity, as no microörganism can be identified by any one character or property.

STUDY OF GROWTH CHARACTERISTICS. (MICROSCOPIC METHOD).

Plate Cultures. In gelatin note the presence or absence of liquefaction in the surrounding medium. Note the shape and character of the liquefaction, if present.

In the agar, no liquefaction takes place. The liquid found on the surface is merely the water of condensation.

The colonies are to be examined at intervals of 24 hours,—with the naked eye, with a hand lens, and under low power microscope or dis-

secting microscope. Distinguish the superficial from the deep colonies and note the characters of the colonies, as to:—

1. **Size.** Diameter at various ages.
2. **Shape. Punctiform** (minute, hemispherical); round; elliptical (oval); irregular (no recognized shape); fusiform (spindle-shaped); cochleate (like snail shell); amoeboid (streaming, irregular); mycelioid (mold-like); filamentous (irregular mass of filaments); floccose (dense, wooly structure); rhizoid (root-like); conglomerate (aggregate of colonies of similar size and form); toruloid (aggregate of colonies like budding of yeast); and rosulate (rosette-like).
3. **Surface Elevation.**
 - (a) **General character of surface as a whole.** Flat (thin, leafy, spread over the surface); effused (spread over the surface as a thin, veilly layer, more delicate than the "flat"); raised (growth thick, with abrupt, terraced edges); convex (surface the segment of a circle but very flatly convex); pulvinate (surface the segment of a circle, but decidedly convex); capitate (surface hemispherical); umbilicate, (having a central pit or depression); conical (cone with rounded apex); and umbonate (having a central convex, nipple-like elevation).
 - (b) **Detailed characters of Surface.** Smooth (surface even); alveolate (marked by depression separated by thin walls so as to resemble a honeycomb); punctate (dotted with punctures like pin-pricks); bullate (like a blistered surface rising in convex prominences, rather coarse); vesicular (more or less covered with minute vesicles due to gas formation, more minute than the "bullate"); verrucose (wart-like, bearing wart-like prominences); squamose (scaly); echinate (beset with

pointed prominences); papillate (beset with nipple or mamma-like processes); rugose (short, irregular folds, due to shrinkage of surface growth); corrugated (in long folds, due to shrinkage); contoured (an irregular but smoothly undulating surface, resembling the surface of a relief map); and rimose (abounding in chinks, clefts cracks).

4. **Internal Structure of Colony.** (Microscopic).

Refraction weak: Outline and surface of relief not strongly defined.

Refraction strong: Outline and surface of relief strongly defined; dense, not filamentous colonies.

(a) **General.** Amorphous (without any definite structure); hyaline (clear and colorless); homogeneous (structure uniform); and homochromous (color uniform).

(b) **Granulations or Blotchings.** Finely granular; coarsely granular; grumose (coarser than the preceding, with a clotted appearance, and particles in clustered grains); moruloid (having the character of a mulberry, segmented, by which the colony is divided in more or less regular segments); and clouded (having a pale ground, with ill-defined patches of a deeper tint).

(c) **Colony Marking or Striping.** Reticulate (in the form of a network, like the veins of a leaf); areolate (divided into rather irregular, or angular spaces by more or less definite boundaries); gyrose (marked by wavy lines, indefinitely placed); marmorated (showing faint, irregular stripes, or traversed by vein-like markings, as in marble); rivulose, (marked by lines like the rivers of a map) and rimose (showing chinks, cracks, or clefts).

(d) **Filamentous Colonies.** Filamentous; floccose (composed of filaments, densely placed); and curled (filaments in parallel strands, like locks or ringlets).

5. **Edges of Colonies.** Entire (without toothing or division); undulate (wavy); repand (like the border of an open umbrella); erose (as if gnawed); irregular (toothed); lobate; lobulate (minutely lobate); auriculate (with ear-like lobes); lacerate (irregularly cleft, as if torn); fimbriate (fringed); ciliate (hair-like extensions, radiately placed); tufted; filamentous and curled.

6. **Optical Characters (after Shuttleworth).**

(a) **General Characters.** Transparent (transmitting light); vitreous (transparent and colorless); oleaginous (transparent and yellow, olive to linseed-oil colored); resinous transparent and brown, varnish or resin-colored); translucent (faintly transparent); porcellaneous (translucent and white); opalescent (translucent, greyish-white by reflected light); nacreous (translucent, greyish-white, with pearly lustre); sebaceous (translucent, yellowish or greyish-white); butyrous (translucent and yellow); ceraceous (opaque and white, chalky); dull (without lustre); glistening (shining); fluorescent and iridescent.

(b) **Chromogenicity.** Color of pigment, pigment restricted to colonies, pigment restricted to medium surrounding colonies and pigment present in colonies and in medium.

Streak or Smear Cultures. In gelatin and agar note the points as indicated under plate cultures. In blood serum note the presence or absence of liquefaction.

Gelatin Stab Cultures. Note as to

1. **Surface Growth.** Same as in plate cultures.

2. **Line of Puncture.** Filiform (uniform growth); nodose (closely aggregated colonies); beaded (loosely placed or disjointed colonies); papillate (beset with papillate extensions); echinate (beset with acicular extensions); villous (beset with short, undivided, hair-like extensions); pulmose (a delicate, feathery growth); arborescent (branched or tree-like, beset with branched hair-like extensions).
3. **Area of Liquefaction** (if present). Crateriform (a saucer-shaped liquefaction); saccate (shape of an elongated sack, tubular, cylindrical); infundibuliform (shape of a funnel, conical); napiform (shape of a turnip); fusiform (outline of a parsnip, narrow at either end, broadest below the surface); and stratiform (liquefaction extending to the walls of the tube and downward horizontally).
4. **Character of the Liquefied Gelatin.** Pellicle on surface; uniformly turbid; granular; mainly clear, but containing flocculi; deposit at apex of liquefied portion.
5. **Production of Gas Bubbles.**

SHAKE CULTURES. Presence or absence of liquefaction; production of gas bubbles; bulk of growth at the surface (aerobic); bulk of growth in depths (anaerobic).

FLUID MEDIA.

1. **Surface of the Liquid.** Presence or absence of froth due to gas bubbles; presence or absence of pellicle formation; character of pellicle.
2. **Body of the Liquid.** Uniformly turbid; flocculi in suspension; granules in suspension; clear, with precipitate at the bottom of tube; coloration of fluid, presence or absence.
3. **Precipitate.** Character; amount; color.

CARBOHYDRATE MEDIA. Note:

Growth; reaction; gas formation.

LITMUS MILK CULTIVATIONS. Note:

Reaction (unaltered, acid or alkaline); odor; gas formation; consistency (un-

altered, peptonized or coagulated); clot (solid, flocculent or ragged and broken up by gas bubbles; coagulum undissolved; coagulum finally peptonized, completely or incompletely; resulting solution, clear or turbid); whey (abundant or scanty, clear or turbid, coagulated by boiling or not).

STUDY OF BACTERIA BY MICROSCOPIC METHODS.

LIVING BACTERIA: Note motility or non-motility. If the organism is one which forms spores observe—spore formation and spore germination.

METHODS OF EXAMINATION.

1. Ordinary Method.

If specimen from solid media is used, a drop of water is placed on a clean slide.

If specimen from liquid media is used, a drop of the media containing the bacteria is used.

(a) Flame the cotton plug of tube containing the culture; extinguish the burning cotton.

(b) Hold test tube containing culture between thumb and finger of left hand.

(c) Hold platinum needle between thumb and forefinger of right hand, and sterilize by heating red hot. Allow to cool.

(d) Remove cotton plug with the third and fourth finger; insert needle, and transfer minute portion of the bacterial culture to the slide.

(e) Return plug to tube and sterilize needle.

(f) Place a clean cover glass over specimens and examine first with 1-6 objective, then with 1-12 of oil immersion pushed gently into a drop of cedar oil placed on the cover glass. Use the fine adjustment. Examination should be made by dim light.

During the examination, stains and other reagents may be run in under the coverslip. The non-toxic basic dyes for "intra-vitam" staining of bacteria are neutral, red, quinoline blue, methylene green and vesuvian in 0.5% aqueous solutions.

2. **Burris' Negative Stain** is sometimes employed to simulate dark ground illumination. It is prepared by mixing 25 cc. of liquid black ink (any liquid waterproof black drawing inks) and 1 cc. of tincture of iodine. Allow the mixture to stand for 24 hours, centrifugalize, pipette off the supernatant liquid to a clean bottle, and then a crystal of thymol or 1 drop of formalin as a preservative.

With a sterilized loop place one drop of the liquid ink close to one end of a slide; sterilize the loop and place a drop of the fluid culture (or emulsion of solid culture) on the slide by the side of the ink; mix thoroughly; sterilize the loop; with another slide spread the mixture across the slide (by placing the end of the slide used as a spreader transversely and at an angle of about 60° on the mixture and allow the fluid to spread across the slide and fill the angle of incidence; draw it toward the end); dry in the air and examine with $1/12$ oil objective.

3. **"Hanging drop" method.**

- (a) Paint a ring of vaseline around the hollow in a "culture slide."
- (b) Place bacterial culture in a small drop on a clean cover glass.
- (c) Invert slide over the cover glass, the drop to be within the vaseline ring, but not to touch its sides, and press down so as to seal tight.
- (d) Invert carefully and examine. This method is for demonstration of bacterial motility. It may be kept for examination from day to day so that spore formation and germination can also be studied.

To Study Spore Formation. Prepare the hanging drop from vegetative forms, add a trace of 0.5% magenta solution to render bacilli more distinct, place slide under microscope (using a warm stage if necessary); with the $1/6$ lens select a bacillus for observation, then substitute the $1/12$ oil immersion and observe the formation of the spore.

To Study Spore Germination. Prepare the hanging drop from old cultivations in which no living vegetative forms are present and observe the process of germination as in "spore formation."

FIXED AND STAINED BACTERIA.

Bacteria are rendered more prominent by the use of dyes and by their aid, note—

1. **The points in morphology, as:—**
Shape, size and pleomorphism if present, record—predominant character of the variant forms, the media on which they are observed, at what period of development).
2. **The details of structure, as:—**
Flagella (if present, record—method of staining, position, arrangement and number); spores (if present, record—method of staining, shape, size, position within cell, condition as to shape of parent cell, optimum medium and temperature, age of cultivation, condition of environment as to temperature and atmosphere, methods of germinations); involution forms (if present, record—method of staining, character e. g. living or dead), shape, on what medium observed, age of medium and environment); metachromatic granules (if present, record—method of staining, character of granules, number of granules and color of granules).

REACTION OF STAINS.

1. **Gram's Method.** Positive (not decolorized) or negative (decolorized.)
2. **Neisser's Method.** If granules are present, record their position and number.
3. **Ziehl-Neelsen's Method.** Acid-fast or decolorized.
4. **Simple Aniline Dyes.** Record those giving best results.

STRAINING METHODS.

Most bacteria stain easily and are therefore easily decolorized.

Some bacteria can withstand alcohol and some withstand strong solutions of mineral acids without decolorizing.

It is often necessary to use heat or a mordant in order that the stain may penetrate the cell.

Potassium hydrate, aniline oil, alcohol, carbolic acid (1-5%) and acetic acid (1-5%) are the common mordants used.

Formulas of Staining Solution.

1. **Simple aniline stains** are prepared by saturating alcohol with methylene blue, dahlia, fuchsin, Vesuvian, gentian violet or thionine.

To these **stock solutions** alcohol may be added from time to time, always allowing an excess of undissolved stain to remain on the bottom of the vessel.

When required for use add 5 cc. of the saturated alcohol solution to 95 cc. of distilled water and filter.

The methylene blue stain is the only one that is permanent. The others must be made fresh as required for use.

All stains should be filtered before using, unless otherwise specified.

2. **Aniline-Gentian Violet.**

Aniline Water.....10 parts.
(Aniline oil 5 cc. and distilled water 100 cc. are well shaken together and filtered. Make fresh every time).

Saturated alcoholic solution of gentian violet1 part.

3. **Aniline-fuchsin.**

Aniline water (see Aniline Gentian V.)10 parts

Saturated alcoholic solution of fuchsin1 part.

4. **Alkaline Methylene Blue.**

(a) **Loeffler's.**

Sat. alc. sol. methylene blue30 parts.

Sol. potass. hydrate (1-10,000)100 parts.

(b) **Kochs.**

Sol. potass. hydrate (10 per cent) 0.2 part.

Sat. alc. sol. methyl. blue 1.0 part.

Water (distilled)200.0 parts.

5. **Carbolic acid solutions.**

(a) **Kuhne's Methylene-blue.**

Methylene blue 1.5 gm.

Abs. alcohol 10.0 cc.

Carbolic acid solution
(1-20) 100 parts.

Stain for 5 minutes.

(b) **Ziehl's carbol fuchsin.**

Basic fuchsin 1 part.
Abs. alcohol 10 parts.
Carbolic acid solution
(1-20) 100 parts.

Filter.

6. **Gram's Iodine Solution.**

Iodine 1 part.
Potass. iodid 2 parts.
Distilled water 300 parts.

7. **Gabbet's Acid Blue (a rapid stain).**

25 per cent solution of
sulphuric acid 100 parts.
Methylene blue 2 parts.

Allow dilute acid to stand 24 hours
before adding the methylene blue.

8. **Unna's Borax Methyl Blue.**

Borax 1 part.
Methyl blue 1 part.
Water 100 parts.

9. **Unna's Polychrome Methylene Blue.**

Potassium carbonate ... 1 part.
Methylene blue 1 part.
Water 100 parts.

Must be ripened for months.

10. **Nicolle's Carbol-thionine.**

Sat. sol. thionine in alc. (90
per cent) 10 cc.
Aqueous sol. ac. carbol. (1
per cent) 100 cc.

Stain sections one-half to one min-
ute.

Contrast Stains.

11. **Eosin Aqueous Solution.**

Eosin (aqueous) 1 gm.
Water (distilled) 100 cc.
Absolute alcohol 5 cc.

12. **Eosin Alcoholic Solution.**

Eosin (alcoholic) 0.5 gm.
Alcohol (70%) 100 cc.

13. **Safranin Aqueous Solution.**

Safranin 0.5 g.
Water (distilled) 100 cc.

14. **Neutral Red Aqueous Solution.**

Neutral red 1.0 gm.
Water (distilled) 100 cc.

15. **Vesuvium (or Bismarck Brown).**

Vesuvium 0.5 gm.
Water (distilled) 100 cc.

Special Stains.

16. **MacConkey's Stain (for capsules).**

Dahlia 0.5 gm.

Methyl green (crystals)... 1.5 gm.
 Water (distilled)100 cc.
 Mix well in mortar, then add
 Fuchsin (sat. alcohol sol.).. 10 cc.
 Water (distilled) to make..200 cc.

17. **Muir's Mordant (for capsules).**

Mercuric bichloride (sat. aq. sol.)2 cc.
 Tannic acid (20% aq. sol.)...2 cc.
 Potash alum. (sat. aq. sol.)..5 cc.

18. **Ribbert's Stain (for capsules).**

Acetic acid (glacial)..... 12.5 cc.
 Alcohol (absolute) 50.0 cc.
 Water (distilled)100.0 cc.

Warm to 36° C. and saturate with dahlia.

19. **Muir's Mordant (for flagella).**

Tannic acid (10% aq. sol.)..10 cc.
 Mercuric bichloride (sat. aq. sol.) 5 cc.
 Alum. (sat. aq. sol.)..... 5 cc.
 Carbol fuchsin (Ziehl)..... 5 cc.

Allow to settle for a few hours, decant off the clean fluid into tubes and centrifugalize.

It will keep for a couple of weeks, but is at its best 4 or 5 days after its manufacture. Must be centrifugalized each time before use.

20. **Loeffler's Mordant (for flagella).**

Tannin (20% aq. sol.)....10 parts.
 Ferrous Sulphate (sat. aq. sol.) 5 parts.
 Decoc. of logwood (1 to 8 aq. sol.) 3 parts.
 Carbolic acid (1% aq. sol.) 4 parts.

Must be freshly prepared.

21. **Loeffler's Stain (for flagella).**

Methylene-blue4 gms.
 Aniline water (freshly saturated and filtered).....100 cc.
 or—

Methylene-violet4 gms.

Freshly saturated and filtered

Aniline water (freshly saturated and filtered).....100 cc.
 or—

Fuchsin4 gms.
 Aniline water (freshly saturated and filtered).....100 cc.

22. **Pitfield's Mordant (for flagella).**

Tannic acid1 gm.
 Water10 cc.

23. **Pitfield Stain.**

Alum. (sat. aq. sol.).....10 cc.

- Gentian violet (sat. alc. sol.). 1 cc.
 Water (distilled)..... 5 cc.
24. **Van Ermengem's Fixing Fluid** (for flagella).
 Osmic acid (2% aq. sol.)....10 cc.
 Tannic acid (20% aq. sol.)...20 cc.
 Acetic acid (glacial)..... 1 cc.
 Prepare a few days before using.
 Filter when needed. It should be violet in color.
25. **Van Ermengem's sensitising Solution** (for flagella).
 Silver nitrate (0.5% aq. sol.)
 Keep in the dark and filter just before using.
26. **Van Ermengem's Reducing solution** (for flagella).
 Gallic acid 5 gms.
 Tannic acid 3 gms.
 Potassium acetate (fused)*10 gms.
 Water (distilled)350 cc.
 Prepare fresh and filter.
27. **Bunge's Mordant** (for flagella).
 Tannic acid (20% aq. sol.)..10 cc.
 Ferrous sulphate (sat. aq. sol.) 5 cc.
 Fuchsin (sat. alc. sol.)..... 1 cc.
28. **Pappenheim's Corallin, Methylene-blue Solution** (for *B. tuberculosis*).
 Corallin1 gm.
 Methylene-blue (sat. alco. sol.)100 cc.
 Glycerine 30 cc.
29. **Spengler's picric acid alcohol**.
 Alcohol (absolute)20 cc.
 Picric acid (sat. aq. sol.)....10 cc.
 Water (distilled)10 cc.
30. **Neisser's Stain** (for diphtheria)
SOLUTION I.
 Methylene-blue 1 gm.
 Alcohol (96%) 20 cc.
 Dissolve and add
 Water (distilled)950 cc.
 Acetic acid (glacial)..... 50 cc.
SOLUTION II.
 Vesuvian 2 gms.
 Water (distilled)1000 cc.
31. **Modified Niesser's Stain** (for diphtheria).
SOLUTION I.
 Methylene-blue (sat. alc. sol.) 4 cc.
 Acetic acid (5% aq. sol.).. 96 cc.
SOLUTION II.
 Neutral red2.5 gms.
 Water (distilled) 1000 cc.

32. **Ehrlich's Haematoxylin** (tissue staining).

I. { Haematoxylin 2 gms.
 { Alcohol (absolute) 100 cc.

II. { Ammonium alum 2 gms.
 { Water (distilled) 100 cc.

Mix I. and II. stand for 48 hours, then filter and add

III. { Glycerine 85 cc.
 { Acetic acid (glacial) 10 cc.

Expose to the light for one month then filter.

33. **Mayer's Haematin** (tissue staining)

I. { Haematin 1 gm.
 { Alcohol (90% warmed to

II. { 37° C.) 50 cc.
 { Potash alum. 50 gms.
 { Water (distilled) 100 cc.

Pour the two solutions slowly and simultaneously into a flask by means of a large funnel to insure thorough mixing.

34. **Mayer's Alum Carmine** (tissue staining).

Alum 2.5 gms.

Carmine 1 gm.

Place in a beaker on a sand bath and add successive small quantities of distilled water; keep mixture boiling for 20 minutes. The solution should make up to 100 cc. Filter.

35. **Picrocarmine.** (tissue staining).

Picrocarmine 2 gms.

Water (distilled) 100 cc.

TECHNIQUE FOR ORDINARY STRAINING.

- (a) Prepare clean cover glass and slide.
- (b) Place drop of water on glass or slide.
- (c) Transfer with sterile needle a minute portion of culture to the drop of water and spread evenly over surface of glass.
- (d) Allow film to dry.
- (e) Fix by passing the glass 3 times through a Bunsen flame.
- (f) Cover specimen with a stain. Allow it to stain from 2 to 10 minutes.
- (g) Wash in water.
- (h) Dry and mount in balsam.
- (i) Examine with 1-12 oil immersion

—use Abbe condenser. If specimen is good, label and preserve.

TECHNIQUE FOR DIFFERENTIAL STAINING.

1. Gram's Method. (Gram's stain).

This is a differential stain the value depending upon the mycoprotein of certain bacteria forming with aniline dyes and an iodid, a compound insoluble in alcohol. Such organisms are said to "stain by Gram" or to be "Gram positive."

- (a) Stain specimen for 5 minutes in aniline gentian-violet.
- (b) Wash in water.
- (c) Stain with Gram's iodine solution for 1 minute, or until the film is black or dark brown.
- (d) Wash in 95 per cent alcohol until no more color comes away.
- (e) Dry and contract-stain in Bismarck-brown (2-3 minutes) or eosin (1 minute).

This step may be omitted when organisms are in pure culture.

- (f) Wash, dry and mount.

The important bacteria retaining the stain are (gram positive).

Smegna bacillus
 Anthrax bacillus
 Tubercle bacillus
 Tentani bacillus
 Leprae bacillus
 Diphtheria bacillus
 Rhinoscleromatis bacillus
 B. Aerogenes capsulatus
 B. Botulinus
 B. Subtilis
 Staphylococcus
 Streptococcus
 Pneumococcus
 Micrococcus tetragenus
 Urethra-coccus.

The important bacteria that decolorize are (gram negative).

Gonococcus
 Displacoccus intracellularis
 B. Mucosus capsulatus
 Bacillus typhoid
 Bacillus coli
 B. Enteritidis
 Bacillus mallei
 Bacillus influenza
 B. Proteus
 B. Morax-Axenfeld
 B. Malignant oedema

B. *Pyocyaneus*
 Bubonic plague bacillus
 Koch-Weeks's bacillus
 Cholera-asiatica spirillum
 Micrococcus catarrhalis
 Paratyphoid bacillus
 Dysenteric bacillus
 Fecal alkaligenes bacillus

2. **Gram-Claudius Method.**

- (a) Stain in methyl violet (1% aq. sol.) for 3 to 5 minutes.
- (b) Treat twice with picric acid (sat. aq. sol.)
- (c) Wash in water and dry.
- (d) Decolorize with clove oil.
- (e) Wash in xylol.
- (f) Mount in xylol balsam.

3. **Gram-Weigert Method.**

- (a) Stain for 5 minutes with aniline gentian violet.
- (b) Wash in water.
- (c) Stain with Gram's iodine solution for 1 minute or until the film is black or dark brown.
- (d) Wash in water and dry in air.
- (e) Wash in aniline oil (1 part) and xylol (2 parts) until no more color come away.
- (f) Wash in xylol.
- (g) Mount in xylol balsam.

4. **Ziehl-Neelson Method** (for B Tuberculosis and other acid-fast bacilli).

- (a) Prepare films as usual.
- (b) Stain in carbol-fuchsin, steaming, but not boiling, for 5 minutes; cool for 25 minutes.
- (c) Wash in 25% sulphuric acid for 3 to 5 seconds.
- (d) Wash in water (Faint red color returns).
- (e) Wash in alcohol till no more color comes away.
- (f) Wash in water.
- (g) Counterstain in weak methylene blue.
- (h) Wash in water, dry, and mount.

The lepra bacillus and the smegma bacillus also stain by this method. The B. lepra stains quickly; the B. Smegmatis is decolorized by the alcohol.

5. **Pappenheim's Method.** (Supposed to differentiate between B. tuberculosis and other acid-fast micro-organisms).

- (a) Prepare films as usual.

- (b) Stain in carbol-fuchsin without heat for 3 minutes.
- (c) Without washing in water treat the film with 3 or 4 successive applications of Pappenheim's (corallin) stain.
- (d) Wash in water.
- (e) Dry and mount.

6. Neisser's Method (for B. diphtheria).

- (a) Prepare films as usual.
- (b) Treat with solution I, (Neisser's stain) for 1 to 3 seconds.
- (c) Wash in water.
- (d) Treat with solution II, (Neisser's stain) for 3 to 5 seconds.
- (e) Wash, dry and mount.

By this method the body of the organism is stained brown and the oval polar granules are blue.

7. Modified Neisser's Method (for B. diphtheria).

- (a) Prepare films as usual.
- (b) Treat with solution I, (Modified Neisser's stain) for 2 minutes.
- (c) Wash in water.
- (d) Treat with Gram's iodine solution for 10 seconds.
- (e) Wash in water.
- (f) Treat with solution II, (Modified Neisser's stain) for 30 seconds.
- (g) Wash, dry and mount.

This must be used on cultivations grown upon blood serum, incubated at 37° C. for from 9 to 18 hours.

The body of the organism is stained a light red and the granules are black.

8. Hunt's Method (for diphtheria).

- (a) Prepare films as usual.
- (b) Treat with aqueous methylene-blue for 1 minute.
- (c) Wash in water and dry.
- (d) Treat with tannic acid (10% solution) for 1 minute.
- (e) Wash in water and dry.
- (f) Treat with an aqueous solution of methyl-orange for 1 minute.
- (g) Wash, dry and mount.

9. Gram's Method with addition of Bismarck-brown (for gonococcus).

- (a) Prepare film with the urethral pus and fix.
- (b) Treat with aniline gentian violet; stain for 15 seconds.
- (c) Wash in water.

- (d) Treat with Gram's iodine solution and permit to remain for from 1 to 2 minutes.
- (e) Wash specimen in 70 per cent alcohol until but a faint violet color remains.
- (f) Stain for 2 minutes with sta. alc. sol. of Bismarck-brown.
- (g) Wash in water, dry and mount in balsam.

By this method the gentian violet stains all bacteria present, but the treatment with the iodine solution and alcohol decolorizes the gonococcus, while the other bacteria in the urethra remain violet.

The addition of the Bismarck-brown stains the previously decolorized gonococcus a light brown. Nuclei of pus and epithelial cells are stained a mahogany color, while the bodies of cells are somewhat lighter in color.

10. **Wheal and Chown (Oxford) Method** (for actinomyces).

- (a) Prepare films as usual.
- (b) Stain with Ehrlich's haematoxylin till nuclei are a faint blue after washing with tap water (examine with microscope).
- (c) Stain in hot carbol-fuchsin for 5 to 10 minutes.
- (d) Wash in tap water.
- (e) Decolorize in alcohol.
- (f) Dehydrate in alcohol.
- (g) Clear in xylol.
- (h) Mount in xylol balsam.

Can also be used for sections.

TECHNIQUE FOR CAPSULE STAINING.

1. **John's Method.**

- (a) Prepare films as usual.
- (b) Warm in a 2% solution of gentian violet till steam arises.
- (c) Wash, dry and mount.

2. **Welch's Method.**

- (a) Prepare films as usual.
- (b) Flood with acetic acid (2%) for 2 minutes.
- (c) Remove acetic acid by means of filter paper or blow it off with a pipette.

(d) Treat with aniline gentian violet for 5 to 30 seconds.

(e) Wash, dry and mount.

3. Hiss' Method.

(a) Prepare films as usual.

(b) Treat with a mixture of gentian violet or fuchsin (5 cc.) and distilled water (95 cc.) heated until it steams.

(c) Wash in a solution (20%) of cupric sulphate crystals.

(d). Wash, dry and mount.

4. Ribbert's Method.

(a) Prepare film as usual.

(b) Treat with Ribbert's stain for 1 to 2 seconds.

(c) Wash, dry and mount.

5. MacConkey's Method.

(a) Prepare film as usual.

(b) Treat with MacConkey's stain for 5 to 10 minutes.

(c) Wash thoroughly, dry and mount.

6. Muir's Method.

(a) Prepare film as usual.

(b) Treat with carbol-fuchsin, warm until steam begins to rise and allow stain to act for 30 seconds.

(c) Wash quickly with methylated spirit.

(d) Wash in water.

(e) Treat with Muir's mordant for 5 seconds.

(f) Wash in water.

(g) Treat with methylated spirit for 30 seconds (film should now be a pale red).

(h) Wash in water.

(i) Stain with aqueous solution of methylene-blue for 30 seconds.

(j) Wash in water.

(k) Dehydrate in alcohol.

(l) Clear in xylol and mount.

TECHNIQUE FOR SPORE STAINING.

1. Single Stain.

(a) Prepare film as usual, except that you pass film through the flame 15 or 30 times instead of the usual three. This will destroy resisting power of spore membrane and permits the stain to reach the interior.

(b) Stain with methylene-blue or fuchsin.

(c) Wash, dry and mount.

2. Double Stain.

- (a) Prepare film as usual (flame 3 times).
- (b) Treat with carbol-fuchsin, steaming for 20 minutes.
- (c) Wash in water.
- (d) Decolorize in acid alcohol (97 cc., 70% alcohol and 3 cc. hydrochloric acid) for a few seconds, in 2 part alcohol and 1 part of 1% acetic acid, or in 1% sulphuric acid.
- (e) Wash in water.
- (f) Examine under the 1/6 objective, (film mounted in water).

The spores should be red and the rods unstained or faintly pink.

- (g) Counter stain with weak methylene-blue for 3 to 4 minutes or gentian violet for 1 minute.
- (h) Wash, dry and mount.

3. Abbott's Method.

- (a) Prepare film as usual.
- (b) Treat with Loeffler's alkaline methylene-blue, heat carefully till steam arises and allow hot stain to act for 1 to 5 minutes.
- (c) Wash in water.
- (d) Decolorize in a solution made up of 1 part of 2% nitric acid and 98 parts of 80% alcohol.
- (e) Wash in water.
- (f) Counter stain in eosin (1% aq. sol.)
- (g) Wash, dry and mount.

4. Mueller's Method.

- (a) Prepare film as usual.
- (b) Treat with absolute alcohol for 2 minutes, then in chloroform for 2 minutes. (This dissolves out any fat or crystals that might retain the spore stain).
- (c) Wash in water.
- (d) Treat with a 5% aqueous solution of chromic acid for 1 minute.
- (e) Wash in water.
- (f) Decolorize in 5% aqueous solution of sulphuric acid for 5 seconds.
- (g) Wash in water.
- (h) Counter stain with Kuehne's carbolic methylene-blue for 1 to 2 minutes.
- (i) Wash, dry and mount.

TECHNIQUE FOR FLAGELLA STAINING.

Bacteria should be from smear agar cultures, 12 to 18 hours old if incu-

bated at 37° C., 24 to 30 hours if incubated at 22° C.

In preparing the films a small quantity of the growth is removed by means of the platinum loop and transferred to a few cc. of distilled water in a watch glass. Gently mix the bacteria with the water by moving the loop to and fro, without touching the side of the watch-glass. Flame a cover slip and spread a thin film, using no force. Dry in air, protect the film from dust. Hold the cover slip between finger and thumb, and fix by passing 3 times through the flame.

1. **Loeffler's Method.**

- (a) Prepare film as described above.
- (b) Treat with Loeffler's mordant, hold it high above the flame and heat, steaming for 1 minute.
- (c) Wash in water (distilled) and dip carefully in absolute alcohol. Wash again in water.
- (d) Filter on to the film a few drops of Loeffler's "flagella stain" and warm as before for 1 minute.
- (e) Wash, dry and mount.

2. **Bunge's Method.**

Same as Loeffler's, except, that Bunge's mordant is substituted for Loeffler's.

3. **Pitfield's Method.**

- (a) Prepare film as described above.
- (b) Mix equal parts of Pitfield's mordant and stain, boil the mixture, and while still hot immerse the film in it for 1 minute.
- (c) Wash in water.
- (d) Examine in water; if satisfactory, dry and mount.

4. **Muir's Modified Pitfield Method.**

- (a) Prepare film as described above.
- (b) Treat with Muir's mordant, hold it high above the flame and heat, steaming for 2 minutes.
- (c) Wash in water and dry carefully.
- (d) Treat with Muir's stain (for flagella) and warm as before for 2 minutes.
- (e) Wash carefully, dry and mount.

5. **Van Ermengem's Method.**

- (a) Prepare film as described above.
- (b) Treat with Van Ermengem's fixing solution, heat carefully, steaming for 5 minutes.
- (c) Wash in water.

- (d) Wash in absolute alcohol.
- (e) Wash in distilled water.
- (f) Treat with Van Ermengem's "sensitizing solution" for $\frac{1}{2}$ to 1 minute; remove excess of fluid with filter paper.
- (g) Transfer film to a watch-glass containing Van Ermengem's "reducing solution" for $\frac{1}{2}$ to 1 minute; remove excess of fluid with filter paper.
- (h) Treat again with the "sensitizing solution" until film commences to turn black.
- (i) Wash in distilled water, dry and mount.

TECHNIQUE FOR STAINING BACTERIA IN TISSUES.

This is practically the same as preparing tissue for histological study. Small pieces of tissue are selected and

Fixed in alcohol (most used; formalin, Zenker's fluid or Mueller's fluid are also used but are not so good as the alcohol fixative).

Hardened, unless alcohol is used as fixative; if not, then the tissues must be kept for 24 hours in 50%, 75%, 90% and absolute alcohols.

Dehydrated, by transferring the tissues to fresh absolute alcohol.

Cleared by xylol or chloroform.

Embedded in paraffin, (Celloidin is also used but is not preferable).

Sectioned. Sections are floated on slide which has been lightly smeared with a mixture of equal parts egg albumin and glycerine to which is added a crystal of camphor or a drop or two of carbolic acid. It is now put aside in the incubator (or warming chamber) for 4 or 5 hours.

Stained by

Loeffler's Method.

1. Dissolve out paraffin with xylol.
2. Remove xylol by flushing section with absolute alcohol.
3. Stain in alcoholic methylene-blue solution for 5 to 15 minutes, or in Loeffler's alkaline methylene blue for from 1 to 24 hours.
4. Wash in 1-1000 solution of acetic acid for about 10 seconds.

5. Treat with absolute alcohol for 10-20 seconds.
6. Clear in xylol.
7. Mount in balsam.

Gram-Weigert Method. (To stain Gram positive bacterial).

1. Dissolve out paraffin with xylol.
2. Remove xylol by flushing section with absolute alcohol.
3. Stain in alum carmin for about 15 minutes.
4. Wash thoroughly in water.
5. Filter aniline gentian violet solution on to the section and allow to stain for about 25 minutes.
6. Wash thoroughly in water.
7. Treat with Lingol's iodine until section ceases to become any blacker.
8. Wash thoroughly in water.
9. Treat with a mixture of equal parts of aniline oil and xylol until no more color comes away.
10. Wash thoroughly with-xylol.
11. Decolorize and dehydrate with absolute alcohol until there remains only a very faint bluish tint.
12. Clear with xylol.
13. Mount in balsam.

The fibrin and hyaline tissue are stained deep blue while Gram positive bacteria appear a deep blue violet color.

To Stain Acid fast Bacteria.

1. Prepare sections for staining as above.
2. Stain with haematin solution 10 to 20 seconds, to obtain a pure nuclear stain.
3. Wash in water.
4. Stain with carbol fuchsin for from 20 to 30 minutes at 47° C.
5. Wash in water.
6. Treat with aniline hydrochlorate, 2% watery solution, for from 2 to 5 seconds.
7. Decolorize in 75% alcohol till section appears free from stain (15 to 30 minutes).
8. Dehydrate with absolute alcohol.
9. Clear with xylol.
10. Mount in balsam.

To Stain Actinomyces.

Mallory's Method.

1. Prepare sections for staining as above.

2. Stain with a saturated watery solution of eosin for 10 minutes.
3. Wash in water.
4. Apply aniline gentian violet for from 2 to 5 minutes.
5. Wash in normal saline solution.
6. Apply Weigert's iodine solution (Iodine 1 part, K. I. 2 parts and water 100 parts) for 1 minute.
7. Wash in water and blot.
8. Clear in aniline oil.
9. Wash in several changes of xylol.
10. Mount in balsam.

STUDY OF CHEMICAL PRODUCTS OF GROWTH (BIOCHEMICAL METHODS).

Effect of Physical Agents on Growth Study of Disinfectants.

1. TEST FOR THE PRESENCE OF ENZYME PRODUCTION.

(a) Proteolytic by

Preparing cultivations in flask bulk (50 cc.), using blood serum and milk serum filtered through porcelain; incubate; after which the liquid is made faintly acid (acetic acid 1%) and boiled; a precipitate of unaltered proteins is thrown down. Filter. Mix 10 cc., of the filtrate and 1 cc. of caustic soda (30%) in a test tube; add, drop by drop, of copper sulphate solution (0.5%). A pink color which becomes violet as copper sulphate is added = proteose and peptone. Saturate the rest of filtrate with ammonium sulphate. The precipitate = proteose.

Filter and divide the filtrate into 3 parts.

- (1) In one part use excess of caustic soda (30% aq. sol.) to displace the ammonia from the ammonium sulphate, then add drop by drop of the copper sulphate solution (0.5%), — a pink color = peptone.
- (2) Boil second part with Millon's reagent (a solution of mercuric nitrate in water containing free nitrous acid, — a pink color = peptone.

(3) Add to the 3rd part some glyoxylic acid solution, then run in sulphuric acid (cong), —

A violet ring at upper level of acid = tryptophane.

(b) **Diastase**, by preparing inosite-free bouillon tube cultivations and incubate. Add equal parts of the cultivations and a thin starch paste (made with water and starch to which is added 2% of thymol); incubate the mixture and incubate at 37° C. for 6 to 8 hours. Filter. Test the filtrate for sugar, using "Fehlings test"—a yellow or orange precipitate = sugar.

(c) **Invertase**, by preparing inosite-free bouillon tube cultivations and incubate. Mix equal parts of the cultivation and carbolized sugar solution (carbolic acid 2 parts, cane sugar 2 parts and water 96 parts) in a test tube; allow to stand for several hours. Filter. Test the filtrate as in the Diastase.

(d) **Rennin**, by preparing inosite-free bouillon tube cultivations and incubate. Heat the cultivation to 55° C. for 30 minutes (to sterilize). With a sterile pipette run 5 cc. of the cultivation into each of 3 tubes of litmus milk. Incubate at 22° C., and examine each day for 10 days.

Absence of coagulation = absence of rennin ferment.

Fermentation Reactions are made upon peptone water containing 2% respectively of each of the following:—a monosaccharide (dextrose), disaccharide (lactose), trisaccharide (mellitose), polysaccharide (dextrin) and glucocide (amygdalin); also 1% respectively of each of the following organic salts:—sodium citrate, formate, lactate, maltate and tartrate. Make tube cultivations in each of the above, observe from day to day for 10 days and note growth, reaction, gas production.

2. TEST FOR THE PRESENCE OF ACID PRODUCTION.

(a) **Quantitative.** Prepare glucose bouillon cultivation in bulk (100 cc.) in a flask; also "control" flask of medium. Incubate both flasks. Heat in Arnold for 30 minutes to sterilize. Determine the titre of the "inoculated" and "control" medium; the difference between the titre gives the total acid production of the bacterium in terms of normal NaOH.

(b) **Qualitative.** Prepare glucose or lactose bouillon cultivation, in bulk (500 cc.), in a litre flask and add 10 gms. of sterilized precipitated chalk. Incubate. Put a cube (about 1 cc.) of paraffin into the cultivation and connect it up with a condenser. Distill over 200 to 300 cc. This distillate (**1st distillate "A"**) will contain alcohol, etc., (see Alcohol production). The **first residue "a,"** will contain the volatile and fixed acids. Filter the first residue ("a") and make up the filtrate (**first filtrate "a"**) with distilled water to 500 cc. and divide into 2 parts. Treat 250 cc. (1st portion of filtrate "a") with 20 cc. phosphoric acid (cong.) to liberate the volatile acids and distill (**second distillate "B"**) to small bulk. The second distillate ("B"), may contain formic, acetic, propionic, butyric and benzoic acids. Add baryta water till alkaline and evaporate to dryness. Add 50 cc. absolute alcohol, allow to stand, stirring frequently, for 2 or 3 hours.

Filter and wash with alcohol.

Filtrate (2nd "b") may contain barium propionate, barium butyrate. Evaporate to dryness and dissolve (2nd filtrate "b") with 150 cc. water. Acidify with phosphoric acid and distill (2nd "b"). Saturate the distillate with calcium chloride and distill over a few cc. — (third distillate "c"). Test dis-

tillate for butyric acid (add 3 cc. alcohol and 4 drops sulphuric acid cong. Smell of pineapple—butyric acid).

Residue (3rd "C") may contain barium acetate, barium formate, barium benzoate. Evaporate off alcohol and dissolve up residue on filter in hot water and neutralize.

Divide the solution into 4 portions.

- (1) Add ferric chloride solution (4% aq.). Brown color = acetic or formic acids; buff ppt. = benzoic acid.
- (2) Add silver nitrate solution (1% aq.), then 1 drop ammonia water and boil; black ppt. of metallic silver = formic acid.
- (3) Evaporate to dryness; mix with equal quantities of arsenious oxide and heat on platinum foil; unpleasant smell of cacodyl = acetic acid.
- (4) Add a few drops of mercuric chloride solution in a test tube, and heat to 70° C., precipitate of mercurous chloride which is slowly reduced to mercury = formic acid.

Second residue ("b"). Wash from filter paper, dissolve by heating with dilute hydrochloric acid (25%) and add calcium chloride solution and ammonia until alkaline.

White precipitate insoluble in acetic acid = oxalic acid.

2nd Portion of first filtrate ("a") (2nd 250 cc.) should be examined for (the ether soluble acids) lactic, oxalic, succinic, benzoic, salicylic, gallic and tannic acids by evaporating the filtrate to a thin syrup, acidify strongly with phosphoric acid. Extract by agitation in a separatory funnel with 5 times its volume of ether. Evaporate the ethereal extract to a thin syrup. Add 100 cc. of water and mix. Add a slight excess of sodium carbonate to a small portion of the mixture and evaporate to dryness on a water-bath, dissolve in 5 to 10 cc. of pure sul-

phuric acid, add 2 drops of copper sulphate (sat. sol.) place in a test tube and heat in boiling water-bath for 2 minutes; cool, add 2 or 3 drops of thiophene solution (0.15 cc. in 100 cc. alcohol) and warm gently. Cherry red color = lactic acid. If, on the addition of the sulphuric acid, a brown color is produced, another sample should be taken and boiled with animal charcoal before evaporating.

If lactic acid is present, prepare zinc lactate by boiling part of the solution of the ether extract with excess of zinc carbonate, filtering and evaporating to crystalize. The crystals obtained have a characteristic form, and if dried at 110° C., should contain 26.87% of zinc.

Test a part of the rest of the solution of the ether extract for oxalic acid by heating with dilute hydrochloric acid and adding calcium chloride solution and ammonia until alkaline. White precipitate insoluble in acid = oxalic acid. Neutralize the remainder and add ferric chloride solution (4% aq. sol.). Red brown gelatinous precipitate = succinic acid. Buff precipitate = benzoic acid and other acids related to benzoic acid. Violet color = salicylic acid. Inky black color precipitate = gallic or tannic acid.

3. TEST FOR THE PRESENCE OF AMMONIA PRODUCTION.

Prepare cultivation in bulk (100 cc.) in a 250 cc. flask and incubate together with a control flask.

After incubation add 2 gms. of calcined magnesia to each flask, then connect up with condensers and distill. Place 50 cc. of the distillate from each in a Nessler tube and add 1 cc. 16 gms. HgCl_2 in 500 cc. pure water, 35 gm. K. I. in 200 cc. pure water. Pour HgCl_2 solution into K. I. solution until faint show of excess is indicated. Add 160

gms. KOH. Dilute to 1000 cc. and add a strong solution of HgCl_2 until red mercuric iodide just begins to be prominent. Let excess of mercuric iodide settle to bottom. Reagent should have a pale straw color to each tube. Yellow color = ammonia. The depth of color is proportionate to the amount present.

4. TEST FOR THE PRESENCE OF ALCOHOLIC PRODUCTION.

Prepare glucose or lactose bouillon cultivation in bulk (500 cc.) in a litre flask and add 10 gms. of sterilized precipitated chalk. Incubate. Put a cube (about 1 cc.) of paraffin into the cultivation and connect it up with a condenser. Distill over 200 or 300 cc. Divide the distillate into 4 portions and test for the production of alcohol, acetaldehyde, acetone. Add Lugol's iodine, then a little NaOH solution and stir till the color of the iodine disappears. Pale-yellow crystalline precipitate of iodoform (with characteristic smell) appearing in the cold, = acetaldehyde, or acetone; appearing only on warming = alcohol. The precipitate may be absent, though odor is pronounced. Add Schiff's reagent (sulphuric acid 3 parts and a 10% solution of ferricchloride, 1 part). Violet or red color = aldehyde.

Add to 10 cc. of the solution, 2.5 cc. of a 25% sulphuric acid, and a crystal or two of potassium bichromate and distill. Reduction of the bichromate to a green color and a distillate, which smells of acetaldehyde and reacts with Schiff's reagent = presence of alcohol in the original liquid. Add a few drops of sodium nitroprusside liquid solution (5%), add ammonia to make alkaline, then saturate with ammonium sulphate crystals. Acetone gives little color on the addition of ammonia, but after the addition of ammonium

sulphate a deep permanganate color is found, which takes 10 minutes to reach its full depth. Aldehyde gives a carmine red not changed by the ammonium sulphate.

5. TEST FOR THE PRODUCTION OF INDOL, (a product of putrefaction).

Make several peptone water test tube cultivations and incubate. Allow the culture to cool to room temperature and remove 2 cc. of the cultivation by means of a sterile pipette, transfer to clean tubes then add 2 cc. paradimethylamino-benzaldehyde solution (paradimethylamino-benzaldehyde 4 gms., absolute alcohol 380 cc.) add 2 cc. potassium persulphate solution (sat. aq. sol.). A delicate rose-pink color appearing throughout the mixture which deepens slightly on standing indicates indol.

A method used for the test in several laboratories is by the ordinary nitrosoindol reaction. Inoculate several tubes, each containing 10 cc. of glucose-free bouillon or peptone water, allow the culture to grow for 5 to 10 days. To each tube of the culture add 10 drops of pure concentrated sulphuric acid, and then 1 cc. of a sodium nitrate solution (.2%). If a pink color develops within 10 minutes, indol is present. In recording the production of indol it is necessary to state the age of the culture, since indol may be produced in 10 days and not in five days.

The reaction may appear immediately, or a faint reaction may appear after a long standing.

The test can also be performed in one stage by making a mixture of concentrated commercial sulphuric, hydrochloric or nitric acid, all of which hold a trace of nitrite in solution. A red color within 20 minutes presence of indol.

6. TEST FOR THE PRODUCTION OF PHENOL (a product of putrefaction).

Prepare a 50 cc. nutrient bouillon cultivation in a 100 cc. Erlenmeyer flask and incubate. After incubation add 5 cc. of sulphuric acid solution (25%), connect up with a condenser, distil over 15 to 20 cc. Divide the distillate into 3 portions. To one portion add 0.5 cc. of Millon's reagent. Digest one part by weight, of mercuric chloride with 2 parts, by weight, of nitric acid sp. gr. 1.42 and dilute the resulting solution with 2 volumes of water and heat to boiling. Red color = phenol.

To another portion add 0.5 cc. of a ferric chloride solution (1% aq. sol.). Violet color = phenol. (If the distillate is acid the result will be negative).

To another portion add strong bromine water.

A crystalline white ppt. of tribromo-phenol or a turbidity = phenol.

In recording the presence or absence of phenol in cultures, the age of the culture and temperature of growth should be stated. If indol and phenol appear together in the same culture, it is well to separate them before making the tests, by the Hoppe-Deyler method, in the following manner: Make a 200 to 300 cc. mosite-free bouillon flask cultivation and incubate, after which, render definitely acid with acetic acid and connect up with a condenser. Distill over 50 to 70 cc. The distillate will contain both indol and phenol. Render the distillate strongly alkaline with caustic potash and redistill. The distillate will contain indol (make test for same) while the residue will contain phenol. When the residue is cold, saturate it with carbon dioxide and redistill. Test this distillate for phenol.

7. TEST FOR THE PRODUCTION OF REDUCING AGENTS.

Color destruction. Prepare tube cultivations in nutrient bouillon faintly colored with litmus, rosolic acid, neutral red, and incubate. Examine the cultures from day to day and note whether any color change occurs.

Reduction of Nitrates to Nitrites.

Prepare tube cultivations of nitrate bouillon on nitrate peptone and incubate together with uninoculated controle tubes. This is necessary as the medium may take up nitrates from the atmosphere and an opinion as to the absence of nitrates in the cultivation is based upon an equal coloration of the medium in the controle tube. Test both the culture and the control for the presence of nitrates by adding drops of sulphuric acid (25%) to the tubes, then run in 2 or 3 cc. metaphenylenediamine (5% aq. sol.) into each tube. Brownish-red color = nitrites. The depth of color is in proportion to the amount present.

8. TEST FOR THE PRODUCTION OF PIGMENTS.

Make tube inoculations upon the various media and incubate at 37° and 20° C. temperatures; grow them also under aerobic and anaerobic atmospheres; also, exposed to and protected from light. Note the conditions most favorable to the formation pigments. Note the solubility of the pigment in hot and cold water, alcohol, ether, chloroform, benzol and carbonbisulphide. Note the effect of acids and alkalies upon the cultivations or upon solutions of the pigment.

9. TEST FOR THE PRODUCTION OF GAS.

Inoculate fermentation tubes filled with sugar bouillons and incubate. Examine at intervals of 24 hours and mark the levels of the fluid when the evolution of gas has ceased, measure the

length of the column of gas with a millimeter scale. Express this column as a percentage of the entire length of the closed branch.

To roughly determine the relative proportions of CO_2 and H_2 —fill the bulb of the fermentation tube with caustic soda solution; close the mouth of the bulb with a rubber stopper; invert the tube 10 times, that the soda may be brought into intimate contact with the gas; return the gas to the end of the closed tube, and measure. The loss in volume of gas = carbon dioxide.

The residual gas = hydrogen. Transfer the gas to the bulb, apply a light and cause it to explode.

Sulphuretted Hydrogen.

Inoculate tubes of iron peptone or lead peptone media and incubate together with control tubes. Examine every 24 hours for several days. The liberation of H_2S will cause the yellowish-white precipitate to darken to a brownish-black or jet black, the depth of color being in proportion to the amount of H_2S present.

STUDY OF THE BIOLOGY OF CULTURES BY PHYSICAL METHODS.

The growth and development of cultures are to be examined under conditions of

1. **Atmosphere.** Prepare 4 sets of cultivation in:—

- (a) Slanted glucose formate agar and incubate aerobically at 37°C ., slanted glucose formate gelatine and incubate aerobically at 20°C . Seal the cultures in Buchner's tubes according to Buchner's method.
- (c) Slanted glucose formate agar; glucose formate bouillon. Grow anaerobically by placing them in Bulloch's apparatus according to Bulloch's method and incubate at 37°C .
- (d) Slanted glucose formate gelatin; glucose formate bouillon. Grow

anaerobically by placing them in Bulloch's apparatus according to Bulloch's method and incubate at 20° C.

Make observations upon the cultivations both microscopically and microscopically at intervals of 24 hours, until the completion of 7 days incubation. By this method it can be determined as to whether an organism is an obligate aerobe, a facultative anaerobe or an obligate anaerobe.

A rough estimate of the above may be made by an observation of the growth in fermentation tubes. If there is a growth in the closed arm as well as in the bulb of the tube, it indicates that the organism is a facultative anaerobe; while a growth occurring only in the bulb or in the closed arm shows that it is an obligate aerobe or anaerobe respectively.

In addition to the observation of growth in the presence or absence of oxygen it is often necessary to observe the growth in—**Gases other than Oxygen** (SO_2 , N_2O , NO , CO_2 , etc.)

Prepare tube cultivations upon solid media and place them in Bulloch's apparatus. Replace the contained air with the selected gas and incubate under optimum temperature.

Examine the cultivations at 24 hour intervals for 7 days, by removing a tube from the apparatus each day.

If no growth is visible, incubate the tube under optimum conditions of temperature and atmosphere, by which the length of exposure to the gas necessary to kill the organism is determined.

2. Temperature.

(a) **Test the range of temperature (minimum and maximum).**

Prepare 10 tube cultivation in fluid media of optimum reaction. Arrange a series of incubators with fixed tempera-

tures varying from 5° C. to 50° C. (Water baths can be used if incubators are not available). Incubate one tube aerobically and one anaerobically in each incubator. Examine at $\frac{1}{2}$ hour intervals for from 5 to 18 hours. Note the temperature (optimum) at which the growth is first observed.

Continue the incubation for 7 days. Note the extremes of temperature at which growth takes place (minimum and maximum temperatures)

(b) Test for the (absolute) optimum temperature.

Prepare 10 tube cultivations in fluid media of optimum reaction. Arrange a series of incubators with fixed temperatures varying 1° C. for 5 degrees on either side of the optimum observed in testing for the range of temperature, and incubate a tube in each incubator. Examine at $\frac{1}{2}$ hour intervals and note the temperature ("absolute optimum") at which growth is first observed.

(c) Test for the Thermal Death-point. Vegetative Forms.

Moist t. d. p. is the temperature which kills a watery suspension of the organism after an exposure for 10 minutes.

Make the test by preparing tube cultivation on solid media of optimum reaction, and incubate for 48 hours. Examine cultivations microscopically to determine the absence of spore; place 3 loopfuls of the surface growth in each of 12 test tubes containing 5 cc. salt solution; mix the organisms through the salt solution; transfer the mixture from each tube into a sterile 250 cc. flask and mix; place 5 cc. of this mixture into each of 12 sterile test tubes and number them consecutively; regulate a water bath to 40° C. and suspend one tube in it so that the upper level of

the bacterial suspension in the tube is about 4 cm. below the surface of the water in the bath, and the bottom of the tube is about the same distance from the bottom of the bath; suspend and adjust a "control tube" containing 5 cc. of salt solution, under similar conditions, plug the tube with cotton and pass a thermometer through the plug so that its bulb is immersed; watch the thermometer in the test until it records 40° C. Note the time and at the end of 10 minutes at this temperature, remove the bacterial suspension and cool it by placing the lower end of the tube in running water. Make 3 gelatin or agar plates, containing respectively 0.2, 0.3 and 0.5 cc. of the suspension, and incubate; place the remaining 4 cc. of the suspension into a flask containing 250 cc. of bouillon and incubate. Observe the cultivations from day to day for 7 days, at the end of which time "no growth" can be recorded.

The same technique is to be carried out in the remaining 11 tubes, varying the conditions so that each tube will be exposed to a temperature 2° C. higher than the immediately preceding one (i. e., 42° C. 44° C. 46° C. 48° C., etc.)

Note the lowest temperature at which no growth takes place up to the end of seven days' incubation, = the thermal death-point.

Dry t. d. p. is that temperature which kills a thin film of the organism after an exposure for 10 minutes.

Make, under sterile conditions, an emulsion with three loopfuls from an optimum cultivation in 5 cc. normal salt, and with the microscope determine the absence of spores. If spores are absent, make 12 cover-slip sterile films and place each in a sterile

Petri dish to dry. Expose each dish in the hot-air oven for ten minutes at temperatures varying 5° C. between 60° C. and 120° C., with a sterile forcep removing each film from the oven as soon as the ten minutes are completed, placing it in a flask containing 200 cc. bouillon; incubate and prepare subcultivations from the flasks showing evidence of growth, to determine that no contamination has taken place.

Spores.

Moist t. d. p. is that time exposure to a temperature of 100 C. necessary to kill all spores present in a suspension.

Make 12 agar slant cultivations and incubate under optimum conditions. Examine microscopically to determine the presence of spores. Under sterile conditions place about 5 cc. normal saline into each tube and by means of a platinum wire emulsify the surface growth; add the 60 cc. emulsion to 140 cc. of normal saline contained in an Erlenmeyer flask; place the flask in a water-bath of boiling water. When the temperature of the flask reaches 100° C. remove by means of a sterile pipette 5 cc. of the suspension from which plate and flask cultivations are made. Repeat process intervals of 25 minutes steaming. Control the experiments by removing the suspension at intervals of $\frac{1}{2}$ or 1 minute during the 5 or 10 minutes preceding the previously determined t. d. p.

Dry t. d. p. Make an agar slant cultivation and incubate under optimum conditions for spore formation. Under sterile conditions place about 5 cc. normal saline into the tube and emulsify the growth. Determine the presence of spores by microscopic examination. Make 12 cover-slip films and place each in a separate Petri dish.

Expose each dish in turn for 10 minutes to a different fixed temperature, varying 5° C. between 100° C. and 160° C.

Complete examination as in the vegetation forms.

REACTION OF MEDIUM.

Range. Make a 24 hour bouillon culture of the organism. Pipette 0.1 cc. of the cultivation into a tube containing 9.9 cc. of sterile bouillon and mix. Inoculate a series of tubes of nutrient bouillon of varying reactions, from + 25 to — 30, viz.: + 25, + 20, + 15, + 10, + 5, neutral — 5, — 10, — 15, — 20, — 25, — 30, with 0.1 cc. of the diluted cultivation and incubate.

Make half hour observations from the third to the twelfth hours and note the tube or tubes in which the growth first appears. (Probably the optimum reaction). At the end of a 48 hour incubation, note the extremes of acidity and alkalinity in which growth has taken place; this indicates the range of reaction."

Optimum Reaction. The steps are indicated in "range reaction," but must be fixed within narrower limits by inoculating a series of tubes which have a variation in reaction of 1 instead of 5 for five points on either side of the tube or tubes in which the growth first appears in the "range reaction."

RESISTANCE TO LETHAL AGENTS.

Desiccation. Make an agar-slant cultivation and incubate for 48 hours, after which examine to determine the absence of spores. Pipette about 5 cc. sterile normal saline into the tube and suspend the growth in it. Make thin spreads of the suspension on sterile cover slips and place them inside of sterile Petri-dishes to dry. When dry, elevate the lids of the Petri-dish in such a way as to allow ventilation and place the dish in a

Muller's desiccator, the upper chamber of which is filled with pure H_2SO_4 , cover with a bell jar, and exhaust the air. At 5 hour intervals admit air, remove a cover slip, and under sterile conditions transfer it to a culture flask. Reseal the desiccator and exhaust the air. Incubate the culture flasks for 7 days if necessary and pour plates from those flasks which show growth to determine the absence of contamination.

Hourly observations should now be made for 5 hours preceding and succeeding the death-time determined above.

Light.

- (a) **Diffuse Daylight.** Make a tube cultivation in nutrient bouillon and incubate for 48 hours. Pour 10 gelatin and 10 agar plates, each containing 0.1 cc. of the bouillon culture. Place one agar and one gelatin plate into the hot and cold incubators, respectively, as controls. On the center of the cover of the remaining plates fasten a black paper, cut in the shape of a cross or some other figure, and expose the plates to the action of diffuse daylight, for 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 hours, after which incubate and examine after 24 and 48 hours and compare with the two controls. If the growth is absent from that portion of the plate not covered by the paper, continue the incubation and daily observations for 7 days.
- (b) **Direct Sunlight.** Prepare as above, except that plates are placed in the direct rays of the sun. Stand a small glass dish (14 cm. \times 5 cm.) on top of each plate and fill with a 2% watery solution of potash alum (to absorb the heat of sun's rays). Make exposure and incubate as in experiment above.
- (c) **Colours.** Test separately with violet, blue, green, red, orange and yellow. Prepare plate cul

tivations as in "light" experiments. Fasten a strip of black paper (3 cm. wide) across the cover of each plate; then paint the colors over the remaining portions of the cover. The colors are prepared by placing 2% of the following dyes:—

Chrysoidine (red), aurantia (orange), naples yellow (yellow), malachite green (green), bluish eosin (blue), and methylviolet (violet), in pure photographic collodion.

Expose the plates to bright daylight for varying periods as in preceding experiments.

Heat. (See thermal d. p.)

Antiseptic and Disinfectants. Bichloride of mercury, formaldehyde, carbolic acid are generally selected for the test, noting the strength of solution and duration of exposure necessary to produce death.

They are examined with reference to

- (a) **Inhibition Coefficient**,—that % of disinfectant present in the medium which is sufficient to prevent the growth and multiplication of bacteria therein.

Prepare a series of 6 bouillon cultivations of an organism in tubes containing 10 cc. of medium. Mark the tubes from 1 to 6; place in No. 1 tube — 2 cc. of a 5% carbolic acid solution (1:100); in No. 2 — 1 cc. (1:200); in No. 3 — 0.6 cc. (1:300); in No. 4 — 0.5 cc. (1:400); in No. 5 — 0.4 cc. (1:500); and in No. 6 — 0.6 cc. (1:1000).

Prepare a series of 6 bouillon cultivations, using

1:1000	1:2000	1:3000	Solution of 1% Hg. Bi- chloride.
0.1 cc.	0.05 cc.	0.03 cc.	
1:4500	1:5000	1:10000	
0.025 cc.	0.02 cc.	0.01 cc.	

Prepare a series of 6 bouillon cultivations, using

1:1000	1:2500	1:5000	Solution of 0.1% For- maldehyde.
0.1 cc.	0.05 cc.	0.03 cc.	
1:10000	1:15000	1:20000	
0.025 cc.	0.02 cc.	0.01 cc.	

Incubate the three sets and examine them from day to day for 7 days and note those tubes, if any, in which growth takes place.

- (b) **Inferior lethal coefficient** — the time exposure necessary to kill vegetative forms suspended in water at 20° to 25° C. in which the disinfectant is present in concentration insufficient to cause plasmolysis.

Prepare 48 hour agar slant cultivations of each of the "test" organisms and examine to determine the absence of spores. Prepare solutions of different percentages of each disinfectant and make a series of hanging-drop preparations from the culture, using the different percentage to prepare the emulsion on each cover slip. Examine these under the microscope to determine the strongest solution which does plasmolyze the organism. Make control preparations of these two solutions and determine the percentage to be tested. Transfer 10 cc. of sterile water into the agar cultures and suspend the growth in it, after which it is transferred to a flask containing 90 cc. of sterile water and well shaken. 10 cc. of this dilution is placed into each of 10 sterile test tubes; one is placed in the 20° C. incubator as a control; to each of the other tubes a sufficient quantity of a concentrated solution of the disinfectant is added to produce the percentage previously determined. Incubate at 20° C. Remove the control tube and one of the other tubes containing the disinfectant at hourly intervals; make subcultivations upon agar and incubate. Examine these cultures from day to day for 7 days and determine the shortest exposure necessary to cause the death of vegetative forms.

- (c) **Superior lethal coefficient**, that time exposure necessary to kill spores suspended in water at 20° to 25° C. in which the disinfectant is present in concentration insufficient to cause plasmolysis.

Make agar-slant cultivations of the "test" organism and incubate under conditions previously determined for the formation of spores.

Employ that percentage solution of the disinfectant determined in the inferior lethal coefficient and complete the investigation as detailed therein, increasing the interval between planting the subcultivations to two, three or five hours if advisable.

Where it is necessary to leave the organism in contact with a strong solution of the disinfectant for a long period of time, all traces of the disinfectant must be removed by several centrifugations and washing of the bacteria with sterile water.

STUDY OF THE PATHOGENICITY OF AN ORGANISM.

For the study of the pathogenicity, the use of living animals has become a necessity in order that the—

Pathogenic properties of bacteria already isolated in pure culture may be determined.

The conditions influencing the virulence of an organism and the pathogenic effect produced by its entrance into and multiplication within the tissues of the body, etc., can only be carried out by animal inoculation.

Raising or "exalting" the Virulence of an Organism.

When it is desired to raise the virulence of a feebly pathogenic organism, one of the following methods of inoculation is necessary—

- (a) A highly susceptible animal is inoculated with a pure culture of the organism and then passed from animal to animal as rapidly

as possible, always selecting the method of inoculation (e. g. peritoneal) which places the organism under the most favorable conditions for growth and multiplication.

- (b) An animal is inoculated with a pure culture of the organism together with a pure culture of some other organism, which is in itself of a virulency sufficient to produce the death of the animal. The inoculation of the two organisms may be made into the same situation or in different parts of the body.

By the association of these two organisms, the one of low virulence will often acquire a high virulence, which may be still further raised by the passage from animal to animal.

- (c) An animal is inoculated with a pure culture of an organism together with an injection of a toxin (e. g., elaborated by the proteins group, etc.) The natural resistance of the animal is lowered, the organism allowed to multiply, its virulence is then raised by passage from animal to animal.

Attenuating or Lowering the Virulence of an Organism.

This is usually brought about by

- (a) Cultivating the organism in a media unsuitable by reason of its composition or reaction.
- (b) Cultivating the organism in suitable media at an unsuitable temperature.
- (c) Cultivating the organism in suitable media, in an unsuitable atmosphere.
- (d) Cultivating the organism in suitable media, under unfavorable conditions as to light, motion, etc.
- (e) Passing the organism through naturally resistant animals.
- (f) Exposing the organism to desiccation.
- (g) Exposing the organism to gaseous disinfections.
- (h) By a combination of two or more of the above methods.

II. Isolation of Pathogenic Bacteria.

Certain parasitic bacteria are with great difficulty isolated from associated saprophytic bacteria by the ordinary culture methods by reason of the difficulty with which they grow upon artificial culture media.

If the parasite and its associated saprophyte are injected into an animal susceptible to the parasite, the pathogenic organism can readily be isolated from the tissues of the infected animal.

Special media may be excellent for the growth of these certain parasites, but the associated saprophytes will also grow so abundantly as to overgrow the parasite; therefore, if the material containing the parasitic organism is inoculated under the skin of a susceptible animal, the pathogenic organism, in a few days, will have entered the blood stream and killed the animal, leaving the saprophytes at the seat of inoculation.

Cultivations made at post mortem from the animal's heart blood will produce a pure growth of the pathogenic organism.

In obtaining the culture from the infected animal, complete asepsis must be the rule. The fur or feathers are drenched with a 2% lysol solution, to prevent the hairs from flying about and entering the body cavities during the autopsy, and also to render innocuous any vermin that may be present. With sterile forceps and scalpel incise and reflect the skin. Sear the whole exposed surface with heated searing irons; remove a part of the body wall with a new set of sterile instruments and proceed to make the culture.

III. Identification of Pathogenic Bacteria.

The morphological and cultural resemblances of certain pathogenic bacteria are in some cases so great as to make identification impossible; the same organism

may under carying condition take on an appearance so different from the typical that results are very confusing. A simple inoculation may decide the point at once.

IV. **Study of the Problems of Immunity.**

It is only by careful study of the behavior of the animal cell and the body fluids together with the infecting organism that we may understand the complex problem whereby the cell successfully resists the invading organism. During the inoculation studies, instances of both racial and individual natural immunity will be met with. Natural immunity, however, is relative only and never absolute and an organism must not be put down as non-pathogenic until many different methods of inoculation have been accomplished upon animals of different species. (See immunity.)

METHODS USED FOR THE STUDY OF THE PATHOGENICITY OF AN ORGANISM:

1. **The living Organism.**

(a) **The Psychrophilic Bacteria.**
(Grow only at or below 18° — 20° C.)

Cultivations are prepared under optimum conditions in nutrient broth, and after seven days' incubation, an amount of the culture corresponding to 1% of the body weight of a frog is injected into the frogs dorsal lymph sac. Observe, if necessary, for 28 days. (See Animal Inoculation).

(b) **Mesophilic Bacteria** (grow at 35° — 37° C.)

Cultivations are prepared under optimum conditions in nutrient broth, and after 48 hours incubation, inoculate a white mouse subcutaneously at the root of the tail, with an amount of the culture corresponding to 1% of its body weight. Inoculate a second mouse of the same weight, or nearly so, ultraperitoneally with a similar dose.

Observe carefully until death occurs, or for 28 days. (See Animal Inoculation).

If death takes place a post-mortem examination is to be made. If death takes place shortly after the injection, the inoculation experiment should be repeated two or three times; and if the organism invariably exhibits pathogenic effects, ascertain, if possible, its minimal lethal dose (from the growth upon solid media) for frogs or white mice, respectively. Now make tests on white rats, guinea pigs and rabbits.

2. **The Toxins.**

Prepare bulk cultivations of the organism in glucose formate broth and incubate for 14 days under optimum conditions. For the determination of:

(a) **Intracellular or Insoluble Toxins.**

Heat the fluid culture in a water bath at 60° C. for 30 minutes. Inoculate a tube of sterile bouillon with an equal amount of the heated culture and incubate under optimum conditions to demonstrate the absence of the living organism.

Inject intravenously that amount of the cultivation corresponding to 1% of the body weight of the selected animal.

Observe during life, or until the 28th day, and in the event of death make a complete post mortem examination.

The experiment should be repeated, and if a positive result is obtained, the minimal lethal dose of "killed culture" is estimated.

(b) **Extracellular or Soluble Toxins.**

Filter the cultivation through a porcelain filter into a sterile filter flask. Inoculate the various animals subcutaneously with a quantity corresponding to 1% of the body weight, and observe, if necessary, for 28 days.

Inoculate a control tube of bouillon and incubate to demonstrate the absence of living organisms.

Repeat the experiment, and if a positive result is obtained determine the minimal lethal dose of the toxin.

ANIMAL INOCULATION.

Animals employed in the study of pathogeneses are the cold blooded frog; toad and lizard; the warm blooded mouse, rat, guinea pig, rabbit and monkey; the hot blooded fowl and pigeon.

Before animals are inoculated they should be carefully examined so as to avoid the employment of any already diseased. This examination should take in the observation of the animal at rest and in motion; the appearance of the fur, feathers or scales, inspection of the eyes, and of the external orifices of the body; tactile examination of the body and limbs, palpation of the groins and abdomen and in many cases microscopical examination of fresh and stained blood-films.

The mouse and rat may suffer from septicemia, the cysticercus form of *taenia murina*; the cystic form of *taenia crassicollis* has its habitat in their lives; scabies; favus and trypanosoma Lewisi.

The guinea pig may suffer from scabies, coccidiosis and tuberculosis. It is well to test the animal by injecting 0.5 cc. of Koch's old tuberculin, which will cause death in those diseased within 48 hours.

The rabbit may suffer from psoric acari. One form (*sarcoptes minor*) first shows itself as yellowish scales and crusts around the nose, mouth and eyes, spreads to the bases and outer surfaces of the ears, to the fore and hind limbs and into the groins and around the genitals. Another form (*psoroptes communis cuniculi*) commences at the bottom of the concha in the form of white, yellowish masses of crusts, scales, feces and dead acari. The coccidium oviforme is a frequent infection. Infection with ordinary pyogenic bacteria frequently occurs in the rabbit.

The monkey is very prone to tuberculosis and should be injected with 1 cc. Old tuberculin.

Anematode (*oesophagostoma inflatum*) resembling the *anchylostomum*, parasitic in cattle, is frequently present in the tissues of the monkey, may bore through the intestinal wall and produce small cysts in the mesentery.

The pigeon may be infected by *haemosporidia* and pigeon diphtheria.

The fowl may suffer from scabies and ringworm, fowl cholera or fowl septicæmia.

Animal inoculation is purely surgical operation, therefore, in its performance strict attention must be paid to asepsis and precautions adopted to guard against contamination of the material to be introduced into the animal.

The Material used for Inoculation may be

1. **Cultures of bacteria grown—**

(a) In fluid media; a definite measured quantity injected by means of a syringe or (if a large bulk is to be introduced) by means of a gravity apparatus consisting of a funnel, rubber tubing and an injection needle.

(b) On solid media; a fluid suspension is made by washing the culture with a little bouillon or normal saline, and then injected as above.

2. **Metabolic products of bacteria (Toxins).** Prepared as previously described and injected as described under cultures of bacteria.

3. **Pathological products** (fluid secretions and excretions, solid tissues) are treated as fluid cultivations. If the material is very thick a small portion of bouillon or normal saline solution may be used to dilute it. Solid tissues are rubbed up in a sterile mortar with a small portion of bouillon.

The Methods of Inoculation.

The animal is held firmly by an assistant or secured to an operating table, liquid soap applied to the area selected for inoculation with a small pad and lathered freely by the aid of warm water; shave thoroughly; wash with 2% lysol solution; wash off lysol with ether and allow the ether to

evaporate; then inoculate by method selected from the following:

1. **Cutaneous Method.** (No anaesthetic).
Make numerous short parallel superficial incisions with the point of a sterile scalpel and when the oozing has ceased, rub the inoculum into the scarifications. Cover the area with a pad of sterile gauze secured by adhesive or collodion.
2. **Subcutaneous Method.** (No anaesthetic if inoculum is solid ethyl chloride spray).
If the inoculum is fluid pinch up a fold of skin between finger and thumb and inject with a hypodermic syringe.
If the inoculum is solid, raise a fold of the skin in a pair of forceps and make a small incision. By means of a probe make a small pocket in the subcutaneous tissue and introduce the tissue inoculum into it. Close the wound in the skin with a clip (Michel's) or a suture and cover the area as in cutaneous method.
3. **Intramuscular.** No anaesthetic if the inoculum is fluid but if solid use A. C. E. anaesthetic. The method is practically the same as in the subcutaneous, except that the injection is made deep into the muscle.
4. **Intraperitoneal.** (No anaesthetic).
For liquid inoculum the method is essentially the same as in the subcutaneous, except that the entire thickness of the abdominal walls is pinched up into a triangular fold. Ascertain that there are no coils of intestine included by slipping the peritoneal surfaces one over the other.
For the solid inoculum, an A. C. E. anaesthetic is used and the aponeuroses between the recti muscle are divided upon a director, the peritoneum likewise, the inoculum introduced; the peritoneum closed with Lembert's sutures; the aponeuroses and skin incision are closed together with interrupted suture.

5. **Intracranial.** (A. C. E. anaesthetic).
 - (a) **Subdural.** By the use of a trephine open the skull in the parietal segment at the point of intersection of the medium line and a line joining the posterior canthi, perforate the dura and with a syringe deposit the material immediately below this membrane carefully so as to produce no injury.
 - (b) **Intracerebral.** Same as in intracranial except that the needle is pushed into the substance of one or the other cerebral hemispheres.
6. **Intraocular.** (Cocaine anaesthetic). Two needles are fitted to a syringe. One is attached to the syringe and the required dose of inoculum is taken into it; the needle is then removed. The other needle is used to pierce the cornea, allowing the aqueous to escape through it, then without removal the syringe is attached and the inoculation is made into the anterior chamber.
7. **Intrapulmonary.** (No anaesthetic). The fluid inoculum is injected through the 5th and 6th intercostal space into the lung tissue.
8. **Intravenous.** (No anaesthetic). The fluid inoculum must be prepared with care in order that when injected a fatal embolism may be obviated. If possible, the fluid should be filtered through sterile filter paper to do away with small fragments of tissue. Eliminate the possibility of air bubbles. After the usual preparation of the skin, plunge the needle of the syringe through the skin into the lumen of the vein and slowly inject the inoculum. Withdraw the needle and press a pledget of cotton over the puncture.

The jugular vein may be utilized in the guinea pig; the posterior auricular vein in the rabbit; the internal saphenous vein in the dog or monkey.
9. **Inhalation** (No anaesthetic). The animal is placed in a closed

metal box and through a hole in one side of it the nozzle of the spraying apparatus (ordinary nasal spray will do) containing the fluid inoculum is introduced and sprayed into the interior of the box. On completion of the spraying, the animal is sprayed thoroughly with a 10% solution of formaldehyde and the animal returned to its cage. The inhalation chamber is thoroughly disinfected. In another method, for both fluid and powdered inoculum, frequently used, a wooden gag provided with a square orifice through which a tracheal or oesophageal tube may be passed down through the larynx into the trachea. Connect the straight portion of a Y-shaped tube to the laryngeal tube; couple one branch of this to a separatory funnel containing the fluid inoculum or insufflator containing the powdered inoculum and the other to a hand bellows. Allow the fluid inoculum to run down into the lungs by gravity, or below the powdered inoculum into the lungs by means of a bellows.

10. **Intragastric.** (No anaesthetic). By use of a gag similar to the one mentioned above, insert a soft rubber catheter into the stomach and allow a measured quantity of the inoculum to run down into the stomach. With some sterile salt solution wash out the last traces of the inoculum in the catheter and then withdraw it.
11. **Feeding.** Pieces of sterilized bread are soaked in the fluid inoculum, or small pieces of tissue inoculum are placed in sterile dishes and offered to the animal.

The possession of pathogenic properties by an organism is indicated by the **infection** of the experimental animal. Infection is considered to have taken place

- (a) When the death of the animal is produced by the inoculum.
- (b) When, without producing death, the inoculum causes local or gen-

eral changes of a pathological character.

- (c) When either with or without death, or the production of local or general changes, certain substances make their appearance in the body fluids which can be shown to exert some specific effect when brought into contact with cultivations of the organism originally inoculated.

The observation upon the animals inoculated must begin immediately and only terminate with the death of the animal. If the animal appears to be unaffected it should be killed at the end of 2 or 3 months and a complete post-mortem carried out.

The examination of the animal should consist of

- (a) **General Observation** daily as to general appearance, weight, and temperature.

- (b) **Special Observations.**

1. As weekly examination of the site of inoculation and the neighboring glands palpated.
2. As to any local reaction (suppuration carefully examined both microscopically and culturally).
3. Frequent examination of the blood histologically.
4. **Examination of the blood bacteriologically** for the presence of the organism previously injected into the animal.

Method:

Sterilize a glass syringe and moisten its interior with a sterile solution of sodium citrate (sodium citrate 10 gm., sodium chloride 0.75 gm., distilled water, 100 cc.) If more than 5 cc. of blood is required, retain about $\frac{1}{2}$ cc. of the sodium citrate solution in the syringe to prevent coagulation of the blood. Prepare the animal and introduce the syringe needle into the lumen of the selected vein; collect sufficient blood; withdraw the needle; deliver the citrated blood into a flask containing 250 cc. of nutrient broth and incubate until growth occurs or until the expiration of 10 days.

5. **Examination of the blood serologically** to demonstrate the presence of antibodies as antitoxin, agglutinin, precipitin, opsonin, and immune body or bacteriolysin. (See under immunization).

Conditions Necessary to Infection are

1. **The micro-organism must be pathogenic.** It must be a parasite.

Organisms that are parasitic are not necessarily pathogenic; however, certain requirements must be met in order that an organism may be infectious for any given animal, and by this is meant, the ability of an organism to live and multiply in the animal fluids and tissues.

Organisms which do not grow at body temperature are not pathogenic, neither are the strictly aerobic organisms as they are not able to obtain oxygen in available combination from carbohydrates. Aerobic organisms are practically unable to multiply in the blood stream and produce general infection.

2. **The organism must be virulent.**

Pathogenic organisms differ very much in their power to incite disease. This variation in virulence occurs not only among different species of pathogenic organisms, but may occur within the same species. Certain organisms when kept upon artificial media or in unfavorable environment for some time, are much less virulent than those isolated from the bodies of man or animal.

3. **The number of organisms which gain entrance to the animal tissue must be of sufficient number.**

A small number of organisms, even though of the proper species and of sufficient virulence, may be overcome by the defenses of the body. The more virulent the organism, the smaller the number necessary to produce disease.

4. **An Infection Path** by which bacteria gain entrance is of importance in determining whether or not disease will occur. Streptococci

when swallowed may cause no effect, while if rubbed into the abraded skin will give rise to a severe reaction. Typhoid rubbed into the skin may not give rise to any reaction of moment, while if swallowed may cause fatal infection. Animals are protected from bacterial invasion by the skin and mucous membrane, and when these are healthy and uninjured, micro-organisms are usually kept out, though they may occasionally pass through uninjured skin and mucosa. There can not be much doubt that the tubercle bacilli may pass through the intestinal mucosa into the lymphatics without causing local lesion.

5. **Animal must be susceptible.**

Susceptibility is relative and not absolute. It may be natural to a certain race; it may be acquired by the presence of conditions which lower vitality; it may be inherited, by reason of an inherited tendency.

Even though virulent pathogenic organisms may pass through an injured portion of the skin or mucosa, it does not necessarily follow that infection will take place, as animals have an immunity (see "Immunity") which, if normally vigorous and active, will usually overcome a certain number of the invading organisms. If this immunity is weak by reason of depression, or the invading microorganisms are very virulent or plentiful, infection takes place.

INFECTIONS.

When microorganisms have gained an entrance into the animal body and give rise to disease, the process is spoken of as infection.

In contact with the body of animals is a vast flora of microorganisms, some constant parasites, some transient invaders, some harmless saprophytes and some capable of becoming pathogenic.

The phenomena of infection are reactions between the microörganism and the body defense.

In order to cause infection bacteria must gain entrance to the body by paths adapted to their own cultural requirements and must be permitted to multiply.

They may then give rise merely to local inflammation, necrosis and abscess formation; they may remain at the point of entrance and elaborate toxins which are absorbed and circulated by the blood; they may, from the local lesion, gain entrance into the lymphatics and blood vessels and be carried freely into the circulation, where, if they survive, **bacteriaemia** or **septicaemia** follows; they may be carried by the blood to other parts of the body and find lodgment in any of the organs and give rise to secondary foci of inflammation, necrosis, and abscess formation (pyemia).

The disease arising as the result of the infection may depend wholly or in part upon the mechanical injury produced by the inflammatory process, the disturbed function caused by the presence of the bacteria in capillaries and tissues, and by the absorption of the products resulting from the reaction between the body cells and the bacteria.

The symptoms characteristic of infectious diseases, to a large extent, result from the absorption of:—

Bacterial Poisons, produced by the organisms themselves.

(a) **Ptomaines** were discovered by Brieger during his investigations into the nature of the poisons evident in bacterial infections. These bodies isolated by him from decomposing beef, fish and human cadavers, although produced from proteid material by bacterial action, and the cleavage products derived from the culture medium, they are not true bacterial poisons in the sense in which the term is now employed.

(b) **Toxins**. The poisons produced by all pathogenic microörganisms are soluble, secretory products of the bacterial cell, passing from the

cell into the culture medium during their life.

They may be obtained free from the bacteria by filtration and in a purer state from the filtrate by chemical precipitation, etc.

The bacillus of diphtheria and the bacillus of tetanus are examples. If a several day bouillon growth of these organisms is passed through porcelain filters, the filtrate will often be extremely toxic, while the residue will be inactive or very weak. If the residue possesses any toxicity at all, the symptoms appearing will be quite different from those produced by the filtrate. Other organisms act in an opposed manner, e. g., spirillum cholera and bacillus typhosus. If these are cultivated and filtered, the filtrate will be toxic only to a slight degree, while their residue may be very toxic. This is evidently due to poisons not secreted into the medium but rather attached to the bacterial body. They are termed **endotoxins**, and the greater number of pathogenic bacteria seem to act under this class.

Mode of action of bacterial poisons is much the same as the ability of the various narcotics and alkaloids to select special tissues or organs and enter into a combination with them, either chemically or physically, or both.

Soluble toxins like the bacillus of tetanus and the botulinus bacillus attack specifically the nervous system. Certain poisons elaborated by certain organisms as the staphylococci, streptococci, etc., attack the red blood cell (haemolysin) while others attack the white blood cell (leukocidin).

These toxins when in solution can be removed by the addition of their specific tissue, e. g., solution of tetanus toxin, if treated with brain substance and centrifuged leaves the solution free from toxin; likewise haemolytic poisons can be removed from solutions by

contact with red blood cells, but only when the red blood cells of a susceptible species are employed.

THEORY OF IMMUNITY.

Several theories have been advanced to account for the various phenomena of immunity. Pasteur advanced the "**Exhaustion theory**," in which bacteria by their growth in the body used up or exhausted something vitally necessary to their subsequent growth.

Retention theory in which certain noxious agents are retained by the body, which prevent further growth of bacteria.

Cellular or biologic theory of Metchnikoff, or "Phagocytosis."

Humoral or chemical theory of Ehrlich, or "Side Chain."

The theory accepted by most bacteriologists is a combination of the theories of Metchnikoff and Ehrlich, and is called the **Cellulo-humoral**.

The theories of immunity, acceptable at the present time, are based upon two branches of study:—

- (1) A conception formulated by the German school under the leadership of Ehrlich, Pfeiffer, Kruse; deal entirely with the phenomena occurring in reaction between bacteria or bacterial products and body fluids.
- (2) The participation of the cellular elements of the body in its resistance to infectious organisms. Phagocytosis was formulated by studies at the Pasteur Institute in Paris, under the leadership of Metchnikoff.

IMMUNITY.

It is plain that the mere entrance of pathogenic organism into the animal body through the skin or mucosa does not necessarily lead to the development of an infection. The body must therefore possess certain means of defense in order that the pathogenic germs after they have gained entrance into the tissue and fluids will

be disposed of, or, at least, be prevented proliferating and elaborating their poisons. The condition which enables the body to accomplish this is spoken of as resistance, and when this resistance is especially marked, it is spoken of as "immunity."

Immunity, therefore, denotes that condition of an organism which enables it to resist an attack of the particular bacteria and their toxic secretion against which they are said to be immune. The varieties of immunity are:—

1. **Natural Immunity**, as an inheritance from immune ancestors.
 - (a) **Species Immunity**.—Many infectious diseases common to man do not occur in animals; e. g. gonorrhea and syphilis do not occur in animals except when produced experimentally and this with the greatest difficulty. Leprosy, influenza, etc., have not been transmitted to animals, likewise human beings are immune to diseases which attack animals.
 - (b) **Racial Immunity**. Separate races, or varieties within the same species, often display differences in their immunities towards pathogenic organisms; e. g., Algerian sheep show a much higher resistance to anthrax than do our domestic sheep. The difference in resistance towards tuberculosis between the Caucasian and the American Indian, the Eskimo and Negro is very striking. Conversely, the comparative immunity of the negro from yellow fever, which shows very great virulence toward the Caucasian.
 - (c) **Individual Immunity** is noticed to some extent in man and may probably be attributed to individual variation in the body metabolism; e. g., depressions in gastric acidity predispose to infection of gastrointestinal origin; anatomical differences may act as predisposing factors towards infection.
2. **Acquired Immunity**.
 - (a) **Active Immunity** is naturally acquired by having had a previous

infection. This is illustrated by an infection with typhoid fever, yellow fever, and many of the exanthemata. A single attack of any of these diseases protects the individual for a limited period and frequently for life.

It may be artificially acquired by:

- (1) Inoculations with weakened, attenuated cultures of bacteria.
- (2) Inoculation with sublethal doses of fully virulent microorganisms. Successive inoculations with gradually increased doses of the virus creates an immunity sufficient to resist ten times the toxic dose.
- (3) Injecting with gradually increased doses of dead microorganism. Used especially in that class of bacteria in which the cell bodies (endo toxins) had been found to be more poisonous than their extra-cellular products (toxins).
- (4) Injecting gradually increased doses of the bacterial product (toxins).

- (b) **Passive Immunity** is acquired by injection of the serum of animals that have been rendered immune by artificial methods, into the individual infected or to be protected.

This type of immunity is used chiefly against diseases caused by bacteria which produce powerful toxins, and the sera of animals immunized against such toxins are called antitoxic sera.

Passive immunity against microorganisms that do not have marked toxin formation has not been successful. The microorganisms which are injurious by reason of the content of the bacteria cell, rather than by the secreted soluble toxins, probably do not produce antitoxins in the sera of immunized animals.

The substances produced by their immunization seem directed against the invading organisms in that they have the power of destroying the specific germ used in the production of immunity.

The anti bacterial sera are used in laboratory animals to immunize them against a large number of germs, and if used just before, at the same time or soon after infection, they seem fairly effective.

In human disease their use has been disappointing, except when the anti bacterial sera could be brought in direct contact with the germs, as in closed cavities or localized lesions; e. g., Flexner's sera for meningitis.

ANTIBODIES.

The treatment of the animal body with bacteria or their products gives rise to a variety of reactions which result in the presence of "**antibodies.**" These bodies are not produced by bacteria or their products only. They may be produced by a variety of poisons of plant and animal origin.

Nuttall, Fodor and Fluggs (1886), noted the bacterial properties of normal blood. A study of the blood sera of immunized animals by Beljarff showed no change from normal as to index of refraction, specific gravity, and alkalinity.

Joachim, Moll, Hiss and Atkinson found immunized blood sera richer in globulin than normal serum.

Very little light was thrown upon the phenomenon of immunity until Nuttall, Fodor and Buchner demonstrated the power of normal blood serum to destroy bacteria. This property of the blood diminished with age and was destroyed by heating to 56° C. Buchner called this themolabile substance **alexin.**

Behring, Kitasato and Wernicke, in 1890 and 1892, showed that the blood sera of actively immunized animals against the toxins of diphtheria and tetanus would protect normal animals against the poisons of these diseases. Behring called this substance, contained in the blood sera of immunized animals, **antitoxins.**

Soon after this Ehrlich produced antitoxin against some of the higher plants. Calmette produced antitoxin

against snake and scorpion poisons; Kempner against the poison of *bacillus botulinus*, etc.

The formation of antitoxins directed against the soluble toxins did not explain the immunity acquired against bacteria which produced no soluble toxin. Pfeiffer (1894), threw light upon this when he injected into the peritoneal cavity of cholera-immune guinea-pigs, cholera spirilla.

The microorganism often underwent complete solution, determined by hanging-drop preparations.

Metchnikoff and Bordet showed that this lytic process would also take place in vitro. The constituents of the blood serum which gave rise to this destructive phenomenon were called **bacteriolysins**.

Gruber and Durham then discovered another specific property of immune serum to which the name **agglutinin** was applied. Certain bacteria, when brought into contact with the serum of animals immunized against them, became clumped, lost their motility and firmly agglutinated.

In 1897, Kraus demonstrated that precipitates were formed when the filtrates of cultures of typhoid, cholera, etc., were mixed with their specific immune sera. These substances he called **precipitins**.

The large variety of substances, some poisonous, some innocuous, that possess the power of stimulating antibody formation in the sera of animals are termed **antigens** or **antibody-producers**.

Ehrlich proposes three forms of receptors in explanation of all varieties of antibodies. (See Side Chain Theory).

1st order haptines or receptors, when free in the circulation, constitute the antitoxins and anti-ferments.

2nd order haptines or receptors, when free in the circulation, serve as anchorage and for the further digestion of antigens. They are precipitins and agglutinins.

3rd order haptines or receptors merely anchor suitable substances and exert no action till combined with the complement. When free

in the circulation with a chemical group having affinity for the antigen and a complementophile group are the amboceptors of bacteriolytic, cytolytic and hemolytic sera.

The second group of receptors which give rise to agglutinins and precipitins Ehrlich believes to be made up of a single hatophore group for the anchorage of the ingested material, and an attached zymophore group, or ferment, which changes the anchored substance preparatory to its absorption by the cell protoplasm.

Bordet has shown that it is not the agglutining itself which agglutinates, but the agglutinin in combination with its antigen is agglutinated by the salt solution. He therefore disagrees with Ehrlich and concludes that the phenomenon of agglutination consists of the union of the antibody with its antigen in a colloidal solution, and that the actual agglutination is a secondary phenomenon depending possibly upon a change in the physical properties of the emulsion. The same he holds to be true of precipitins.

The antibodies which can be demonstrated are agglutinin, precipitin, opsonin and bacteriolysin.

These substances cannot be isolated in purity apart from the blood serum, consequently, methods have been elaborated to permit of their recognition.

The serum from the experimental animal (specific serum) is studied and compared with the serum from an uninoculated animal of the same species (normal serum). In order that the differences existing in the serum of various individuals may be eliminated, a mixture of sera obtained from several normal animals (pooled serum) is usually used.

Collection of Serum.

Shave the dorsal surface of the ear, wash with lysol, remove lysol by dropping ether over it and allowing the ether to evaporate, puncture the vein and collect the blood by means of a small blood-collecting pipette, touch the issuing drop of blood with

one end of the pipette, which is held at an angle so that the blood will flow down into it. When the tube is about two thirds full, hold it by the end containing the blood, the clean end pointing obliquely upward, warm this end with the bunsen flame to expel some of the contained air; then seal it in the flame. Shake the blood into the closed end and seal the other end in the flame.

When the blood has clotted place the pipette in the centrifuge, the end first closed pointing downward, and centrifugalize thoroughly. The blood cells will then be found collected in a firm mass at one end, and above them will appear the clear serum.

Mark the pipette above the serum with a file and break it off at this point; the serum is now accessible for testing.

IMMUNIZATION.

In order to study the pathogenic powers of any particular bacterium, the active immunization of one or more normal animals becomes necessary. This is done by various methods; seldom by one method only, but usually by a combination of methods adapted to suit each particular case. The ordinary methods used are as follows:

1. **Active Immunity.** (See "Immunity"). An illustration of how the general methods of immunization are carried out is as follows:—

A full grown rabbit weighing from 1200 to 1500 gms. or over is most suitable for immunization.

A small rabbit is inoculated intraperitoneally with one or two loopfuls of 24 hour optimum culture of the virulent organism selected. Death should follow within 24 hours, or at most, in 48 hours. Under aseptic precautions the rabbit is "posted," and a loopful of heart blood is transferred to 50 cc. of sterile broth. This is incubated at 37° C. for 24 hours. Also prepare several cultures on optimum media from the heart blood of the rabbit; label them all O. C. (original culture). Incubate at 37° C. for 24

hours, after which seal the mouth of the plug tube of all but one culture with an Indian rubber cap painted with shellac or paraffin, and replace in incubator. (Prevents evaporation and culture will remain virulent for a considerable period of time).

Suspend the 24 hour broth culture in a water bath at 60° C. for one hour in order to kill the culture. Cool immediately. Now determine the sterility of the cultivation by transferring 1 cc. to each of several tubes of broth; incubate at 37° C. for 24 hours. If a growth occurs, heat the culture in the water bath again at 60° C. for 1 hour. Test again for sterility. If sterile, inject the suitable animal mentioned above intravenously with 2 cc. of the killed culture; also inject 10 cc. into the peritoneal cavity. Watch the animal the next few days; it will lose some weight and may show pyrexia.

When the temperature and weight have become normal again, inject a killed culture in a mount of 5 cc. intravenously and 20 cc. intraperitoneally. Weight and pyrexia reaction like but less marked than that following the first inoculation will probably follow.

Subcultivate on optimum medium, the uncapped O. C.; incubate for 24 hours at 37° C.; determine the minimal lethal dose upon a number of mice.

One week after the last killed culture injection, prepare a fresh optimum subculture from another O. C. tube and incubate for 24 hours. Prepare the minimal lethal dose and inject subcutaneously into the abdominal wall. A local reaction, a pyrexia and loss in weight will probably be observed. In about ten days, inject a similar minimal lethal dose into the peritoneal cavity.

Note weight and temperature of animal carefully, regulating the time of the animal's inoculation by its general condition and continue to inject living cultivations into the peritoneal cavity in gradually increased doses by multiple of ten.

At intervals of 2 months the animal's serum is tested for its specific antibodies.

Under favorable conditions, after 6 months' work, the animal may be injected intra peritoneally with an entire optimum cultivation of the organism without any ill effect.

The animal serum, if withdrawn in about a week following the injection, will, if injected in doses of 0.01 cc. into a mouse, protect it against ten times the minimal lethal dose of the organism.

Immunity has been created by reason of the formation of an **antibody** specific to the bacterium in question and was sufficient in amount to destroy enormous doses of the living organism, the antigen in this case being a bacterial protoplasm of the organism with its endotoxin.

If death did not immediately follow the injection of the antigen, specific antibodies are always formed in greater or lesser extent; and in experimental work a sufficient amount of any required antibody may be obtained without carrying the process of immunization to completion.

If the immunization of a rabbit toward bacillus typhosus be carried out along the lines indicated above, it will be noticed that after a few injections of the killed cultivations that the blood serum of the animal contains specific agglutins for the bacillus typhosus, and if the object of the experiment has been directed toward the preparation of agglutinin, the inoculation may be stopped, even though the animal is not yet strictly immune.

Antibodies may also be formed in response to antigens other than micro-organisms, as can be demonstrated by the injection into animals of foreign proteins, such as egg albumin, blood sera or the red blood cells from an animal of different species. This will lead to the formation of specific antibodies possessing affinities for their specific antigen. Hemolysin is a common antibody of this type and is found in the blood serum of an animal that has previously been injected with the washed red blood cells from an animal of a different species. This serum will possess the power of disintegrating the blood cells of

the variety employed as antigen, and cause these red blood cells of any other species of animal.

The action of this serum is due to the presence of two distinct bodies; the one, hemolysin, and the other, complement.

Hemolysin (immune body, copula, sensitizing body, and amboceptor) is a thermostable antibody which is formed by the repeated injection of foreign red blood cells into an animal. Although it is itself inert, it will link up the complement present in normal serum to the red blood cells of the variety used as antigen. A combination of the two results is Hemolysis.

Hemolysin is obtained by collecting fresh blood serum from an animal that has been inoculated with material in question and then exposed to a temperature of 56° C. for 15 to 30 minutes to destroy the complement. It is now spoken of as inactivated serum. It is reactivated by the addition of fresh normal serum which contains the complement.

Although hemolysin is of importance in making clear many problems of immunity, its main and practical importance is in its application of the hemolytic system to certain laboratory methods having for their object the identification of infected entity or the diagnosis of existing infection.

For its use directed towards the methods of laboratory diagnosis, it is very convenient to prepare hemolytic serum specific for human blood. Ox blood, sheep blood, or goat blood may, however, be used instead.

Complement (or alexin) is a thermolabile oxidizable body present in the normal serum of every animal in a variable but unalterable amount. It is a substance which exerts a lytic effect upon all foreign matter introduced into the blood or tissues. It is, in itself, comparatively inert and is capable of exerting its greatest lytic effect only when in the presence of and in combination with a specific antibody or immune body.

It is obtained by collecting fresh blood serum from any healthy normal animal. Guinea pig serum is most frequently employed.

Preparation of hemolytic serum.

Take 2 cc. of citrated human blood collected from a vein aseptically, place it in the centrifuge and centrifugalize it thoroughly. Wash it with normal saline and centrifugalize again. Repeat this procedure twice, after which, by means of a sterile pipette, transfer the washed blood cells into a sterile capsule. Add 5 cc. of normal saline and mix thoroughly. By means of a sterile glass syringe, inject the blood suspension into the peritoneal cavity of a healthy rabbit weighing at least 2500 gms. At the end of seven days, inject the washed blood cells from 10 cc. of human blood mixed with 5 cc. of normal saline. After another interval of 7 days, repeat the injection of washed blood cells from 10 cc. of human blood mixed with 5 cc. of normal saline.

After 5 days, collect about 2 cc. of the rabbit's blood, allow it to clot, separate the serum and transfer it to a sterile test tube. Place the test tube in a water bath of 56° C. for 15 minutes to inactivate the serum, then the serum quantitatively for hemolytic properties as follows:—

Titration of Hemolytic Serum.

1. Two test tubes marked A and B each containing 9 cc. of normal saline.
2. Add 1 cc. of rabbit serum to tube A; mix thoroughly.
1 cc. of this mixture is added to tube B; mix thoroughly.
3. Place ten small test tubes in a rack and number them from 1 to 10.
4. By means of a pipette place into
Tube No. 1. 0.5 cc. of hemolytic serum = 0.5 cc. hemolytic serum.
Tube No. 2. 0.2 cc. of hemolytic serum = 0.1 cc. hemolytic serum.
Tube No. 3. 0.5 cc. of tube A mixture = 0.05 cc. hemolytic serum.
Tube No. 4. 0.3 cc. of tube A mixture = 0.03 cc. hemolytic serum.
Tube No. 5. 0.2 cc. of tube A mixture = 0.02 cc. hemolytic serum.
Tube No. 6. 0.1 cc. of tube A mixture = 0.01 cc. hemolytic serum.
Tube No. 7. 0.5 cc. of tube B mixture = 0.005 cc. hemolytic serum.

- Tube No. 8. 0.03 cc. of tube B mixture = 0.003 cc. hemolytic serum.
Tube No. 9. 0.02 cc. of tube B mixture = 0.002 cc. hemolytic serum.
Tube No. 10. 0.01 cc. of tube B mixture = 0.001 cc. hemolytic serum.
5. To each of the above ten tubes add 1 cc. of the red blood cells suspension.
 6. Add sufficient normal saline to the tubes containing a small amount of material, to bring the columns of fluid to the same level.
 7. Shake each tube so as to thoroughly mix its contents. Plug the mouth of the tube with cotton and place all in the incubator at 37° C. for 1 hour.
 8. Remove the tubes from the incubator and into each tube, by means of a pipette, place 0.1 cc. complement (guinea pig serum); replace tubes in incubator for 1 hour.
 9. Remove the tubes from the incubator and if all the tubes are not completely hemolized, stand on one side, in ice-chest if possible, for 1 hour.
 10. Examine all tubes for—
Complete hemolysis = clear red solution, no deposit of red cells at the bottom of the tube.
Absence of hemolysis = clear or turbid colorless fluid, with a deposit of red cells at the bottom of the tube.

The smallest amount of hemolytic serum causing complete hemolysis is known as the minimal hemolytic dose (M.H.D.) and, if hemolysis has occurred in all of the tubes down to No. 7, the M.H.D. of this particular serum is 0.005 cc. 200 minimal hemolytic doses per cc. This serum is strong enough for experimental work. As a matter of fact, complete hemolysis down to tube No. 6 will indicate a serum sufficiently strong (= 100 M.H.D. per cc.) If the first one or two tubes show complete hemolysis only, the rabbit should receive further injections in order to raise the hemolytic power to the proper high level.

THE STORAGE OF HEMOLYSIN.

If the rabbit serum hemolytic contents is found to be sufficient, chloroform the rabbit and remove aseptically as much blood as possible from the heart and place it in sterile centrifuge tubes. Place the tubes in the incubator at 37° C. for 2 hours, after which times centrifugalize thoroughly. Pipette off the clear serum and fill in quantities of 1 cc. into small pipettes, sealed hermetically, in the blow pipe flame, avoid scorching the serum. Place the small pipette containing the serum, after having been sealed in the water bath at 56° C. for 30 minutes (destroying the complement); i. e., inactivating the serum and at the same time insuring sterility. A longer exposure reduces the hemolytic power. Place the pipette in a metal box and store in the ice-chest.

LYSINS are substances occurring in normal and immune sera which have the power of destroying and dissolving bacteria and dissolving or liberating the hemoglobin of the red blood cells and also have a lytic action on the various body cells.

When acting on bacteria, they are called bacterolysins; on the red blood cells, hemolysins; on the body cells, cytolytins. The mechanism of the process is complex. Certain substances which kill bacteria and the body cells but do not actually dissolve them are spoken of respectively as bactericidal substances and cytotoxins.

Blood cells of one animal, injected into another animal of another species, gives rise to a hemolytic substance in the blood serum of the animal injected, which is specific for the variety of cells injected. These hemolysins are termed **heterolysins**.

Ehrlich and Morgenroth injected the washed red blood cells of one goat into another and found that the serum of the injected goat would, after a time, develop hemolytic power against the blood cells of the goat whose blood cells had been used but did not possess hemolytic power toward the red blood cells of all goats. Such substances producing hemolysins in

members of its own species are called **isolysins**.

The injection of isolysins produced anti-isolysins which were again specific. They were not able to produce substances that would hemolyze the animals own red blood cells (**autolysin**).

Lytic substances can be prepared for a large number of bacteria and for many body cells. These bodies may be increased markedly during the process of immunization. The substances having the power to produce lysins are called lysinogen and are distinct antigens as the lysins are antibodies. The lysins may be prepared by injecting the live cells, the dead cells, the disintegration products of cells and in some cases the metabolic products of cells.

From the fact that the bacteriolytic digestive power of immune serum after being destroyed by heating or attenuated by time can be restored—"reactivated"—by the addition of small quantities of normal blood serum, Bordet concluded that the bactericidal or bacteriolytic action of the serum depended upon two substances. One present in normal serum and thermolabile, he identified as Buchner's alexin. The other, more stable, produced or increased in serum by immunization, he called the "sensitizing substance," which he believed acted upon the bacterial cells and rendered them susceptible to the action of alexin.

Ehrlich called the thermolabile substance or alexin "compliment" and showed that it was always present in normal serum and was very little, if at all, increased during the process of immunization.

The "sensitizing substance" he called the "immune body," and this he showed to be increased during immunization. Ehrlich argued that when bacteria or blood cells were injected into the animal, certain chemical components of the injected substances were united to side chains of protoplasm of the tissue cells. The excessive production of these receptors caused their detachment and subsequent invasion into the circulation as

"immune bodies." These immune bodies must therefore possess atom complexis, which endow it with chemical affinity for the bacteria or red blood cells used in its production.

The complement does not combine directly with the blood cell or bacteria, but does so through the intervention of the immune body which possesses two atom groups or haptophores; one the cytophile haptophore group, possessing strong chemical affinity for the blood cell or bacteria; the other complementophile haptophore group possessing a weaker affinity for the complement,



The Structure of Lysins.

Lysins and bactericidal substances are composed of a thermolabile part—"complement"—which is destroyed at a temperature of 56° to 60° for 30 minutes and a thermolstable part—"amboceptor"—having a double combining power. The amboceptor withstands a 60° C. temperature for 24 hours, but will be destroyed at a temperature of 70° C. Ehrlich believes these amboceptors to be free chemical receptors of the body cells produced by the same method as are the anti-toxins, but differing from them in that they have two combining groups; one called the citophyle group, with which the amboceptor combines with bacteria or other cells; and the other, the complementaphyle group, with which it combines with the complement.

The complement seems to be a normal constituent of the blood sera and other body fluids and is undoubtedly produced by the various body cells, leucocytes, etc. During the immunization of animals, it increases but

slightly, if at all. It is supposed to be made up of two groups, one a haptophore group, which combines with the amboceptor, and another a zymophore group which really produces the lytic action after the haptophore has combined with the amboceptor. On heating the complement, the zymophore group is destroyed and a complementoid is produced. This substance is similar to a toxid and will combine with amboceptor but no lysis will result. (See antitoxins).

It is the amboceptor that is increased during process of immunization. The complement will not combine with the cells unless the amboceptor is present and has first combined with the cells. It is probable that in the body fluids there are many complements which may activate a variety of amboceptors. Various sera have been noted which possess amboceptors for certain cells, but they are not lytic because they do not possess the necessary complement. For example, dog serum contains amboceptors for the anthrax bacterium, but no complement. If, in such cases, a foreign complement, such as guinea pig or rabbit serum is added, there will be lysis of the bacterial cells.

Occasionally the absence of a complement may benefit an animal, and this may account for the seeming natural immunity as illustrated when the venom (this is nothing more than amboceptors) of a poisonous snake is injected into an animal, such as the hog, which possesses no complement, no lysis of the cells takes place. On the other hand, should the animal, such as rabbit or man, possess, as they do, the necessary complement, lysis will take place.

Substances are sometimes normally present in sera which have the power of combining with amboceptors which may be present and in turn, prevent the amboceptors from combining with the cells so that when the complement is added there will be no lysis. These substances are spoken of as **anti amboceptors** and they may be developed in an animal by immunization with amboceptors of definite kind

(anti antibodies). Certain other substances which may also engage the amboceptors but cannot be called anti-amboceptors in the true sense, accomplish the same purpose and are therefore classed with these bodies.

Deviation of the Complement. The complement may be deviated in several ways, and as a result lysis may be prevented. Sometimes there is noted in sera normal substances which combine with the complement and prevent it from combining with the amboceptors. These substances are spoken of as **anti complements** and may be produced by the immunisation of animals with complements.

The complement may occasionally be absorbed by tissue cells and in this way prevented from combining with the amboceptor. In certain cases, where the serum contains an excess of amboceptors and only a small amount of complement, it may be deviated by reason of the fact that the cells will then have taken up all the possible amboceptors, leaving an abundance of free amboceptors in the serum to combine with the complement, so that no lysis will take place. (This theory advanced by Neisser and Wechsberg has created wide discussion but is believed to be erroneous).

Fixation of Complement (as a test for antibodies). The demonstration of this was first worked out by Neisser and Wechsberg and it is now used for testing the sera for unknown antibodies similar to bactericidal substances and lysins. The reaction is made use of in the recently devised test for syphilis. (See Wasserman reaction).

Filtration of Immune Body and Complement.

Filtration of the serum will allow the amboceptor to pass through while the complement is held back. The amboceptor filters equally well, whether or not mixed with the complement.

COMPLEMENT FIXATION.

Test for Immune Body (Amboceptor).

The term "complement fixation" is applied to a method of investigation by which even the small quantities of

any given amboceptor can be demonstrated in the serum. The method was devised by Bordet and Gengou and prepared as follows:

Tube No. 1	Tube No. 2
Bacterial Ambo- cepto (Plague immune serum, heated).	Normal serum, heated.
Plague Emulsion.	Plague Emulsion.
Complement (Fresh Normal serum).	Complement (Fresh Normal serum).
—Allowed to act for 5 hours, then add—	
Hemolytic ambo- ceptor (Heated hemo- lytic serum).	Hemolytic ambo- ceptor. (Heated hemo- lytic serum).
Red blood cells.	Red blood cells.

———— = Results = ————

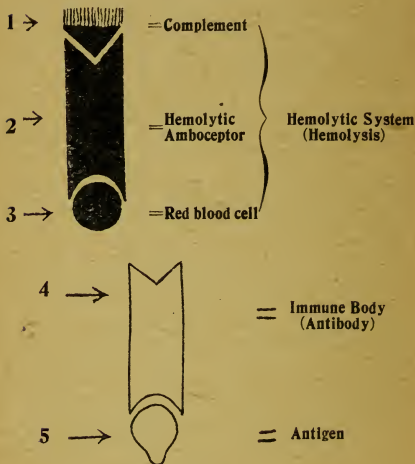
No hemolysis. (Fluid turbid or with red ppt).	Hemolysis. (Fluid clear and red).
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The conclusion to be drawn from this, would be, that in No. 1, the presence of amboceptor had led to the absorption of all the complement and that in No. 2, there being no bacteriolytic immune body to sensitize the bacteria and enable them absorb complement, the complement was therefore left free to activate the added hemolytic amboceptors.

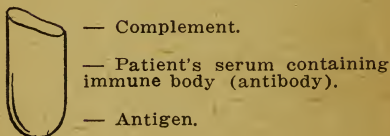
This principle of complement fixation, discovered by Boedet and Gengou in 1901, has been utilized in bacteriological investigations and in the practical diagnosis for the determination of specific antibodies in serum.

The reaction depends upon the fact that when an antigen is mixed with its inactivated antiserum, in the presence of complement, the complement is firmly fixed by the combined amboceptor and antigen in such a way that it can no longer be found free in the

mixture. If this mixture is now allowed to stand at a suitable temperature for an hour or more, then an emulsion of red blood cells and inactivated hemolytic serum are added, no hemolysis will take place as there is no free complement present to complete the hemolytic system. (See e. g. below).



Eg. = Test tube containing—

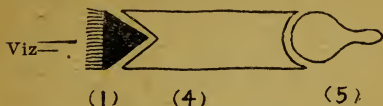


— Placed in incubator at 37.5° for one hour, then add—

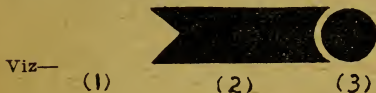
— Hemolytic amboceptor
— Red blood cells.

— = Results = —

No hemolysis occurs by reason of the immune body combining with the complement and antigen—



Allowing the red blood cell and the hemolytic amboceptor to remain free—



No completion of hemolytic system.

If the original mixture contains no antibody for the antigen used, the complement present is not fixed and will be available for the activation of the hemolytic serum. (See (1) (2) (3) above).

The reaction is therefore seen to depend upon the fact that neither antigen alone nor amboceptor alone can fix the complement, but that this fixation is carried out only by the combination of antigen plus amboceptors. Any specific amboceptor can be determined by this method, provided the homologous or stimulating antigen is used and vice versa.

DETERMINATION OF ANTIBODIES BY COMPLEMENT FIXATION.

When testing immune sera for certain amboceptors in man or animals by microorganisms which can be cultivated, either the whole organism or its extracts may be used as antigen.

Bordet and Gengou use a thick salt solution emulsion of a 24 hour agar slant culture of the organism. In the use of tubercle bacilli, 80 mg. of bacilli are emulsified in 1 cc. of salt solution.

Wasserman and Bruck prepare antigen by emulsifying 10 agar slant cultures in 10 cc. of sterile distilled water, after which it is placed in a

shaking apparatus and shaken for 24 hours. 0; 5% of carbolic acid is added and the fluid cleared by centrifugation. The old or the new tuberculins or "Bacillary Emulsion" are used.

Method.

H. S. = Hemolytic serum (heated for 15 min. at 56° C., i. e. inactivated).

Comp. = Complement (Fresh guinea pig serum).

H. R. B. C. = Human red blood cells.

S. S. = Specific serum from inoculated animals, — inactivated.

P. S. = Control "pooled serum" from normal animals of the same species, — inactivated.

Ant. = Antigen (organism grown on solid media and previously having served as antigen in the inoculated animals).

—Place in test tubes—

1	2	3
0.1 cc. Comp.		0.1 cc. Comp.
0.2 cc. S. S.	0.2 cc. S S.	
1.0 cc. Ant.	1.0 cc. Ant.	1.0 cc. Ant.
	4	5
	0.1 cc. Comp.	0.1 cc. Comp.
	0.2 cc. P. S.	
	0.1 cc. Ant.	

—Incubate at 37° C. for one hour—

Add to each tube 1 cc. of H. R. B. C. and 4 minimal hemolytic doses (see titration of hemolytic serum) of H. S.

—Incubate at 37° C. for one hour—

Results

No. 1 — **No hemolysis** = indicates the presence in the serum of the inoculated animal of a specific antibody to the organism used in the inoculations; since it shows that the complement has been bound by the immune body to the bacterial antigen and none has been left free to enter into the hemolytic system.

Hemolysis indicated that no appreciable amount of antibody has yet been formed in response to the in-

oculations; i. e., there is no infection, since the complement remained unfixed at the time of the addition of the H. R. B. C. solution and the H. S.

No. 2 = **No hemolysis** } act as con-
 No. 3, 4, 5 = **Hemolysis** } trol to No. 1.

It may sometimes be convenient to sensitize the H. R. B. C. just before they are needed. This is done before the completion of the first period of incubation.

Method.

Place 5 cc. of H. R. B. C. and 20 minimal doses of H. S. in a sterile test tube and allow them to remain at room temperature for 15 minutes. The red cells are then sensitized and ready for use.

When the tubes are removed from the incubator at the end of the first hour, 1 cc. of the sensitized red cells are added to each tube; mixed thoroughly and the tubes returned to the incubator for the second period.

Complement Fixation for the Determination of Immune Body of Syphilis.

The so called "**Wasserman Reaction**" (although not strictly belonging to the domain of bacteriology) has recently become so prominent as a diagnostic agent in syphilis that an outline of it will be given.

1. Antigen.

Wasserman first made use of salt solution extracts of the organs (spleen, etc.) of a syphilitic fetus, in which the uncombined products (free syphilitic antigens) of *spirochaete pallida* were assumed to be present. He cut the tissue into small pieces and added 4 parts by weight of a normal salt solution containing 0.5% of carbolic acid to 1 part of the tissue. This was placed in the shaking apparatus for 24 hours and then centrifugalized.

The supernatant liquid was used as the antigen. Porges and Meyer prepared antigen by extracting syphilitic organs with alcohol. The syphilitic liver was chopped up and extracted with 5 volumes of absolute alcohol for 24 hours, filtered

through paper and then the alcohol was evaporated in vacuo at a temperature not exceeding 40° C. About 1 gm. of the greenish residue is emulsified in 100 cc. of salt solution containing 0.5% of carbolic acid and filtered through thin paper. The filtrate is used as the antigen.

Noguchi prepared antigen by thoroughly macerating normal liver or spleen in five times its volume of absolute alcohol. Place it in the incubator and allow it to extract for 6 to 8 days with thorough stirring daily. It is then passed through cheese cloth and filtered through paper. The extract is now evaporated to dryness at room temperature and the sticky, brownish residue is dissolved in a small quantity of ether and 4 times its volume C. P. acetone is added. A heavy, sticky, brown precipitate settles to the bottom. This mass is used as the antigen and may be preserved under acetone. The acetone soluble fraction is discarded. When wanted for use, about 0.2 gm. of the mass is dissolved in about 5 cc. of ether, 100 cc. of normal salt solution added and shaken till the ether has evaporated. The antigen is now titrated, and when this is accomplished it is ready for use.

The use of syphilitic organs for the preparation of antigen is not necessary in order to obtain a substance which will combine with the syphilitic immune body.

Many nonspecific antigens will give reasonably reliable results. Porges and Meier found that a 1% commercial lecithin in carbolized salt solution furnished a suitable antigen. This has, however, not been universally accepted.

They also found that normal foetal liver would give good results; likewise others have successfully used an alcoholic extract of guinea pig heart.

The ingredient furnishing the immune body binding power is unknown as yet, although it is claimed to be due to the lipoids.

Antigen must be standard before it can be used for the actual test. The substances used as antigens often have the power, if used in too large quantities of binding the complement. It is therefore necessary to determine the largest quantity of antigen which may be used without binding complement. This may be done by mixing graded quantities of antigen with a constant quantity of complement, in duplicate sets, and adding to each tube of one set 0.2 cc. of a normal serum and to the other 0.2 cc. of a known syphilitic serum. These substances are allowed to remain together for one hour and then red blood cells and inactivated hemolytic serum are added. The quantity which has caused complete inhibition with the syphilitic serum, but none with normal serum, is the one to be used in the subsequent tests.

A dilution of the antigen should be made with salt solution in such a way that 1 cc. shall contain the required amount of antigen. (E. g. if 0.05 cc is wanted, mix 0.5 cc. with 9.5 cc. of salt solution. 1 cc. of this can be used in each tube in the test).

The Hemolytic Serum, Amboceptor.
Prepared as outlined on page 130, using sheep's blood.

By reason of the fact that small amounts of precipitins for sheep's serum may be present in the serum, due to insufficient washing of the corpuscles employed in the immunization, and cause the formation of precipitates which have a tendency to carry down the complement from a mixture, it is therefore necessary that the serum is of high potency in order that the quantities used for the reaction may be as small as possible.

The smallest amount of hemolytic serum that has caused complete hemolysis in 1 cc. of a 5% emulsion of washed blood corpuscles is spoken of as the **hemolytic unit**.

Many make use of two hemolytic units for the actual reaction.

Complement (fresh guinea pig serum) is obtained by anesthetizing a guinea pig, incise the carotid artery and allowing the blood to flow into a large Petri dish. The dish is put away in the ice chest until the serum has separated, which is then carefully removed.

The serum may be centrifugized to insure complete separation from blood cells. The complement in G. P. S. is for practical purposes, constant in quantity. It should be kept, except when in actual use, or a low temperature, and should not be used after 3 days from the time of preparation.

Sheep's Corpuscles are obtained by receiving the blood of a sheep in—

(1) a flask containing glass beads, and shaking thoroughly for about 10 minutes to completely defibrinate the blood. The corpuscles are washed free from the serum by centrifugalization in salt solution

(2) a flask containing a sterile solution of 0.5 % sodium citrate and 0.85% sodium chloride, which will prevent clotting and the corpuscles may be washed free from the citrate solution by centrifugalization in salt solution. Thorough washing with salt solution is essential in order to preclude the occurrence of precipitates and to remove traces of complement. The bulk of the centrifugalized corpuscles is measured and 19 parts of sterile salt solution is added. This forms a 5% emulsion of corpuscles which is the solution employed for the test.

Serum to be tested for Syphilitic antibody.

3 to 5 cc. of blood is removed from a vein, or if conditions will not permit such procedure, the blood may be obtained from the finger or ear. The quantity of blood must always be of sufficient quantity to furnish 1 cc. of clear serum. The serum is now inactivated by heating at 56° C. for 20 to 30 minutes.

Noguchi advises inactivation at 54° C. as the 56° C. destroys the syphilitic antibody in part.

Test.

Into a double row of test tubes place:

Control tube	Control	Control	Test
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
Back row	B	B	B
0.1 cc. Complement.	0.2 cc. known negative normal serum.	0.2 cc. known positive syphilitic serum.	0.2 cc. patients — unknown — serum.
3.0 cc. Salt Solution	0.1 cc. complement	0.1 cc. complement.	0.1 cc. complement.
	3.0 cc. salt solution.	3.0 cc. salt solution.	3.0 cc. salt solution.
<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
Front row	A	A	A
0.1 cc. complement	0.2 cc. known negative normal serum.	0.2 cc. known positive syphilitic serum.	0.2 cc. patients — unknown — serum.
Required amount of antigen.	0.1 cc. complement	0.1 cc. complement	0.1 cc. complement
	—Antigen —	—Antigen —	—Antigen —
2.0 cc. salt solution.	2.0 cc. salt solution.	2.0 cc. salt solution.	2.0 cc. salt solution.

—Place in water bath at 40° C. for 1 hour—

Add to each tube 1 cc. of a 5% solution of sheeps' corpuscles and two units of amboceptor.

—Place in water bath at 40° C. for 1 to 2 hours—

Results.

If test is positive, tubes 3A and 4A will show **no hemolysis** while all other tubes show complete hemolysis.

Tube No. 1. "A" shows active complement. "B" shows antigen alone is not sufficient to deviate complement.

Tube No. 2. "A" shows no deviation of complement in presence of normal serum alone. "B" shows that the particular serum alone will not deviate the complement.

Tube No. 3. "A" shows that antigen is specific in that it deviates complement in the presence of syphilitic antibody. "B" shows syphilitic antibody will not alone deviate the complement.

Tube No. 4. "A" shows the presence of an antibody specific to the antigen employed in that the complement was deviated by antigen in presence of test serum.

Noguchi's Modification of the Wasserman Test.

Anti-human hemolytic amboceptor is used instead of an anti-sheep amboceptor. It is obtained by 4 or 5 injections of washed human corpuscles into rabbits. The amboceptor unit is obtained as in the original Wasserman. Two units are used.

The fact that human serum contains normally no amboceptor active against the human red corpuscle is important and has an advantage over the original Wasserman.

Human serum, normally, may contain a variable quantity of amboceptor for sheep's corpuscles, consequently the actual amount of hemolytic amboceptor used in the original Wasserman is uncertain. This is not so in the Noguchi, as the actual quantity of amboceptor is known exactly by titration.

Antigen. Prepared as in Wasserman.

Complement. A 40% fresh guinea pig serum is made by mixing 1 part of serum with 1.5 parts of salt solution. 0.1 cc. of this solution is used for the test.

Human Corpuscles. Normal corpuscles, or those of the patient himself, may be employed. The patient's corpuscles should not be used for other tests than that on the patient's own serum. 1 cc. of a 1% emulsion of washed corpuscles is used for the test.

Patient's Serum to be tested for the Syphilitic antibody.

Obtained as in Wasserman or in Wright's tube.

About 2 cc. should be taken.

Noguchi Test.

Into a double row of test tubes place:

Control

Control

1

2

3

Back row B

B

B

4

1 gtt. patients—
unknown serum.
0.1 cc. complement
(40% guinea pig
serum.)
Normal saline to
make 1 cc.

1 gtt. known
syphilitic serum.
0.1 cc. complement

1 gtt. known
normal serum.
0.1 cc. complement

0.1 cc. complement
Normal saline to
make 1 cc.
(for hemolytic
system control)

Normal saline to
make 1 cc.

Normal saline to
make 1 cc.

1

2

3

Front row A

A

A

1 gtt. patients
unknown serum.
0.1 cc. complement
Normal saline to
make 1 cc.
Antigen.

1 gtt. known
syphilitic serum.
0.1 cc. complement.
Normal saline to
make 1 cc.
Antigen.

1 gtt. known
normal serum.
0.1 cc. complement.
Normal saline to
make 1 cc.
Antigen.

—Shake, and place in water bath at 38° - 40° for one hour—

Add to each tube two units of amboceptor and the human red blood cell emulsion.

—Shake, and replace in water bath for one hour or more till controls are hemolyzed—

Results.

If test is positive there will be no hemolysis in tubes 1A and 2A while all others are hemolyzed.

Determination of Antigen by Complement Fixation.

In testing for suspected antigen, the procedure is reverse to that of testing for suspected antibodies. The serum or bacterial extract to be tested for antigen is brought into contact with an antibody specific for the antigen in the presence of complement; and at the end of an hour at suitable temperature, free complement is again determined by hemolytic reaction, as in the antibody tests.

Hemolytic Amboceptor. Prepared in the rabbit for sheep corpuscles.

Inactivated and titrated as for Wasserman test.

Two units are used.

Bacterial Antiserum. Prepared by immunizing a rabbit. It must be highly potent. The smallest quantity of the immune serum which will fix the complement in the presence of an emulsion or extract of the microorganism in question is determined by experiment.

The bacterial emulsion is prepared by scraping the growth from 24 hour agar slant cultures, drying it, and macerating in a mortar with salt solution until a slight opalescent emulsion is formed.

Prepare a series of tubes, each containing 0.1 cc. of the bacterial emulsion, 0.1 cc. complement and gradually diminishing quantities of inactivated specific immune serum, ranging from 0.1 cc. downward.

Incubate the tubes at 38° - 40° C. for 1 hour.

Add the required quantities of red blood cells and hemolytic immune serum. The smallest quantity of

immune serum which has completely inhibited hemolysis is the unit. A quantity slightly in excess of the unit is used in the test.

Complement. Fresh guinea pig serum (0.1 cc. used in routine work). It should however be titrated if possible and used in double the quantity necessary to produce hemolysis of 1 cc. of a 5% emulsion of blood cells, in the presence of two units of amboceptor.

Sheep Corpuscles. Prepared as in Wasserman test.

Patient's Serum. Obtained by the usual method and inactivated at 56° C. for 20 minutes.

Test.

Prepare a series of tubes, each containing:

1. Complement, 0.1 cc. or the determined quantity.
2. Antiserum, the determined quantity.
3. Serum to be tested for antigen in diminishing quantities from 1 cc. downward.
4. Salt solution for dilution to 3 cc.
5. Control tubes containing the same ingredients without the antiserum.
6. Incubate for 1 hour at 40° C.
7. Add required quantities of amboceptor and red cells.
8. Incubate again.

Results = A positive reaction if there is no hemolysis in the tubes containing the patient's serum.

Proteid Differentiation by Complement Fixation, such as human or animal blood was shown by Gengou in 1902. The test is said to be more delicate and reliable than the precipitation tests.

Hemolytic amboceptor. Prepared as for Wasserman test.

Complement. Prepared as for Wasserman test.

Sheep Corpuscles. Prepared as for Wasserman test.

Specific Antiserum. Prepared by immunizing a rabbit with the proteid material for which the test is to be made. Filtrate by using diminishing quantities of the antiserum in a series of test tubes containing the determined quantity of com-

plement, and the antigen which is to be tested for, i. e., the homologous serum with which the antiserum has been produced.

The test should be so delicate as to determine 0.0001 cc. of the antigen, consequently this quantity is added to each tube.

The tubes are incubated for 1 hour. The hemolytic amboceptor and red cells are then added.

The unit represents the smallest quantity of antiserum which has completely inhibited hemolysis.

One and one-half to two units are used for the test.

Solution of the proteid material to be tested. Prepare as for precipitin test.

Test.

Prepare a series of tubes containing:

1. Complement, quantity determined by titration.
2. Antiserum, quantity determined by titration.
3. Diminishing quantities of the substance in which the antigen is suspected, ranging from 0.1 cc. downward to 0.0001 cc.
4. Salt solution to make dilution to 3 cc.
5. Control tubes containing the same ingredients without the antiserum.
6. Incubate for 1 hour at 40° C.
7. Add required quantities of amboceptor and red cells.
8. Incubate again.

Results = A positive reaction if there is no hemolysis in the tubes containing the suspected antigen.

EHRlich's SIDE-CHAIN THEORY derives its name from its analogy to what happens in the Benzol ring when its replaceable hydrogen atoms are substituted by "side-chains."

The theory itself is based upon the mechanism of cell nutrition in its relation to the mode of production of specific antitoxins.

In order that a cell may be nourished, the nutritive substance must enter directly into chemical combination with some elements of the cell protoplasm.

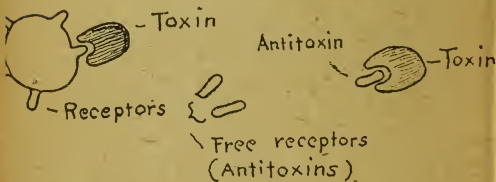
The highly complex protoplasmic molecules of cells are made up of a central

atom-group, upon which the specialized activities of the cell depend, and several outer atom-groups (side-chains) by which the cell entered into chemical relation with food and other substances brought to it by the circulation.

In just the same way the nutritious substances are brought into relation with the cell by means of the side atom-groups, so will also isomeric toxins. These side-chains are called "receptors," and if they have an affinity, by reason of isomerism or chance for a given toxin they unite with the toxin and are therefore rendered useless for their normal physiological function of nutrition.

These receptors are probably cast off and regenerated by the normal reparative mechanism of the body.

The regenerative process does not stop at simple replacement of the cast off elements, but goes on to over compensation, so that they are reproduced in excess of the physiologic needs of the cell, and therefore cast off to circulate in the blood as antitoxins. These receptors (outer-atom-groups, antitoxins) retain their specific affinity for the toxins used in their production and will unite with the poison before it can reach the sensitive cells, and in this way protect the cell from the poison.

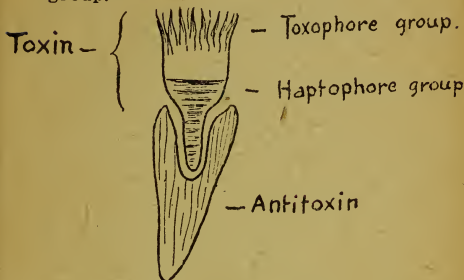


Toxin analysis (Ehrlich).

Toxin solutions deteriorate with time; i. e. a toxin bouillon which contained 80 toxin units per 1 cc. was found to contain but 40 units after 4 or 5 months.

Ehrlich found that such bouillon retained its full original power of neutralizing antitoxin.

The toxin molecule must therefore contain two separate atom-groups. One stable-group, possessing the power of binding antitoxin, he called the "haptophore" or "anchoring" group. The other, the one by which the toxin molecule exerts its deleterious action, is more easily changed or destroyed, he calls the "toxophore," or poison group.



In the toxin-bouillon in which a part of its poison has been lost while the neutralizing antitoxin power still remains, it is quite evident that the toxophore group, or some of the toxin, must have been changed or destroyed. Altered in this way he calls it "toxoid."

Substances found in fresh bouillon, which have a weaker affinity for antitoxin than toxin itself, called "toxins," are primary secretory products of the bacteria.

The "toxoids" are of two kinds—namely, those which have a stronger affinity for antitoxin than toxin itself (protoxoids), and those whose affinity for antitoxin is equal to that of toxin (syntoxoids).

The toxon has a haptophore group similar to that of toxin, but a different toxophore group. It differs from toxin in that it lacks the power to produce acute symptoms; it causes gradual emaciation and paresis in animals.

The toxophore group, producing the harmful results, is divided into toxin, toxoid and toxon.

The haptophore group of the toxin, then, possesses the affinity for the receptor or antitoxin.

The haptophore groups of all three of these substances are alike. In toxid, the toxophore group has been destroyed or altered; in toxon it is qualitatively different from that of toxin. It should therefore produce antitoxins.

AGGLUTININS.

While investigating the Pfeiffer reaction with *B. coli*, Gruber and Durham noticed that if the immune serum was added to bouillon cultures of *B. coli*, the cultures would lose their turbidity and flake-like clumps would sink to the bottom of the tube.

Widal applied this agglutination reaction to the practical diagnosis of typhoid.

Gruber and Durham believed the agglutinins to be identical with the immune body concerned in the Pfeiffer reaction, which injured the bacteria, thereby rendering them susceptible to alexins. It has since been shown that agglutinins and bactericidal substances are in no way parallel. Strongly agglutinating sera may be very weak in bactericidal power and strongly agglutinating sera may be very weak in agglutinating power; the relative quantity of these substances depends upon the method of immunization.

Agglutinated bacteria are not killed by the agglutination and are often as virulent as non-agglutinated cultures. Agglutinins remain active after exposure to over 55° C. temperature. Some will withstand 65° to 70° C. and can not be reactivated by the addition of normal sera. This excludes the participation of complement in this reaction.

The agglutinins do not dialyze.

Normal sera contain small amounts of agglutinin — "normal agglutinins" — probably due to the various microorganisms parasitic upon the animal body.

Agglutinins can be produced by introducing microorganisms subcutaneously, intravenously or intraperitoneally. Almost all the known bacteria will

produce agglutinin, and it will, as a rule, appear in the blood of animals three to six days after the micro-organisms' introduction; increase to a maxim at the 7th to the 13th day, they then fall off till they reach a level, at which they remain for a long time.

Agglutination is not limited to bacteria; just as hemolysins are produced by the injection of red blood cells, so hemagglutins are similarly formed.

Agglutinating Test.

Microscopic Method.

1. Collect a small amount (5-10 drops) of blood in a small glass tube.
2. Separate the serum by centrifugation.
3. By means of Wright's capillary pipette or the white mixing pipette accompanying the hemocytometric counting chamber, dilute the serum with normal salt solution in proportion of 1 to 20. From this, subdilute in proportion of 1-40, 1-80, 1-160, etc., and place each dilution in a separate sterile watch glass.

(Dilutions can also be made by the drop method, using a capillary pipette from which a drop of serum is placed in a watch glass and then normal saline dropped into it till proper dilution is obtained).

4. With the platinum loop, place a drop of the serum from each of the dilutions of serum on a cover glass, and inoculate each with a loopful of a 24 hour old bouillon growth of the organism.
5. Press the cover slips carefully over the chamber of culture slides, the margins of which have been singed with vaseline (hanging drop method).

See that the various dilutions are properly indicated on the slides.

6. Examine immediately under the 1/6 objective of the microscope, and discard the slides if any clumps are observed. If the mount is satisfactory.
7. Set the mount aside and re-examine at the end of $\frac{1}{2}$ hour. If reaction is positive, all the microörgan-

isms will be found motionless and gathered in clumps of variable size.

This will be the case at least in the lowest dilutions, while in the higher ones it may be necessary to wait until another half hour has expired.

The higher the dilution in which complete clumping is obtained, the greater is the diagnostic value.

Macroscopic method.

This test is made in series of small test tubes of 0.5x5 cm. size. In these test tubes 1 cc. of the different serum dilutions and the bacterial emulsion are mixed. They are now placed in the incubator for a few hours and then kept at room temperature.

Hiss has observed that agglutination will be hastened in some instances if after their removal from the incubator, they are placed in the ice chest.

When agglutination takes place, clumps of bacteria are seen to form, which settle to the bottom, much like snow flakes on the tube. The surface of the sediment is heaped up and irregular. The supernatant fluid becomes entirely clear.

When the reaction is negative, the sediment is an even, granular one with flat surface, and the emulsion remains turbid.

PRECIPITINS.

Kraus (1897) demonstrated that sera of animals immunized against a particular microorganism when mixed with the clear filtrate of bouillon culture of the particular organism, would give rise to visible precipitate. In that the precipitate occurred only when the filtrate of a bouillon culture of an organism and the sera of animals immunized with same organism led Kraus to name them "specific precipitates," or precipitin.

Precipitin formation is not limited to bacterial immunization, but is also found like the phenomena of agglutination and lysis. Substances produc-

ing the phenomena in sera are called precipitinogens.

Precipitins like agglutinins may be inactivated by heating to from 60° to 70° C. and cannot be reactivated by adding normal sera, etc. Inactivated precipitin, while unable to produce precipitates, will bind the precipitinogen. This is shown when inactivated precipitin is mixed with precipitinogen no reaction occurs if fresh precipitin is added.

Precipitin is, therefore, like toxin, made up of two atom-groups, a stable haptophore and a labile precipitophore group. It is the opinion of many that precipitins are identical in structure with amboceptors. Just as in agglutins there is in precipitin a certain degree of "group reaction"; that is, the precipitin obtained with a colon bacillus will cause a precipitation with culture filtrates of allied organisms. This may be easily adjusted, however, by the use of proper dilution similar to that used in agglutination tests.

Wasserman and others found other use for this reaction as a means of distinguishing the blood of one species from that of another. Precipitins have not been demonstrated in normal sera.

PRECIPITIN TESTS.

These tests may not only be applied to bacteria, but also to the various proteid substances.

Bacterial Antisera.

1. The bacterial antisera are produced by injecting rabbits by intraperitoneally or intravenously with emulsions of organisms (either broth cultures or salt solution emulsions of agar cultures) in gradually increasing quantities on 5 or 6 occasions, at intervals of from 5 to 6 days. In 7 to 12 days after the last injection, the animal is bled and a preliminary test made as to the precipitating value of the serum.

If this is insufficient, more injections may be made. In 5 to 12 days after the last injection the animal is bled and the sera preserved by sealing in glass bulbs

and kept in the dark at a low temperature. A preservative as chloroform may be added.

The antisera should be absolutely clear. If turbid, it may be filtered through porcelain candles.

2. **The bacterial filtrates for test** are produced by growing the organism in broth composed of 0.5% Liebig's extract of beef, peptone 1%, salts 5% with a reaction of + 5.

The cultures are incubated from a week to several months, and then filtered through porcelain or Berfeld candles until perfectly clear.

The extracts may also be made by emulsifying agar cultures in salt solution and incubating them at 37° C. for a week or more, then filtering.

When the two reagents have been completed, the test is made as follows:—

Mix in a series of narrow test tubes.

- (a) Tube No. 1. — 0.5 cc. antibacterial serum and 1 cc. bacterial filtrate.

Tube No. 2 — 0.5 cc. normal serum and 1 cc. bacterial filtrate.

Tube No. 3. — 0.5 cc. anti bacteria serum and 1 cc. salt solution.

Tube No. 4. — 0.5 cc. salt solution serum and 1 cc. bacterial filtrate.

- (b) Place tubes in incubator at 37° C.

- (c) If test is positive, tube No. 1 shows a haziness, which develops into a distinct cloudiness or even a flocculent ppt. within one hour. Tubes 2, 3, 4 remain clear.

The precipitating antisera against proteid solutions are prepared by methods analogous to those employed for the production of antibacterial sera. If tests are to be made upon proteid material, as blood stains, meat (as detection of horse meat substitution for beef), etc., they should be extracted with salt solution, in an approximate dilution of 1-500.

The solutions are filtered to insure clearness.

To test the unknown proteid with serum of an animal immunized with the proteid sought, mix in a series of narrow test tubes:—

Tube No. 1.—0.1 cc. immune serum and 2 cc. unknown proteid solution.

Tube No. 2. — 0.1 cc. immune serum and 2 cc. known proteid solution of variety suspected (similarly diluted).

Tube No. 3. — 0.1 cc. immune serum and 2 cc. proteid solution of different nature (similarly diluted).

Tube No. 4.—0.1 cc. immune serum and 2 cc. salt solution.

Tube No. 5. — 2 cc. unknown proteid solution.

Test is positive when a precipitate appears in tubes No. 1 and No. 2, but not in any of the others.

ANTITOXINS.

Serum Vaccine Antitoxin.

Antitoxins are produced for all bacteria producing soluble toxin, and for the toxic substances of a large number of other plant and animal cells. They are called antitoxins because they combine with and render inert the soluble toxins. (See side-chain theory). They are labile chemical substances which resist analysis or probably similar to euglobulins, and are composed of molecules of large size. It was at one time supposed that antitoxin was but a toxin in a different form. This, of course, has been disproved. The amount of antitoxin produced is much greater than the amount of toxin which is injected or produced during an infection. The union between a toxin and an antitoxin is of a chemical nature. The union of these two substances forms a compound that is harmless and differs from the toxin and the antitoxin in that it is much more stable. Toxins have a greater affinity for the three haptophile receptors of cells (free antitoxin) than for those still associated with the cells. The toxin and antitoxin always

combine, if possible, before the toxin and the body cells enter into chemical union. In certain cases when the toxin has been bound by the body cells and the antitoxin is produced in sufficient amount or injected, the toxin cell chemical union will be broken up and the toxin and antitoxin will combine. This is illustrated in diphtheria, and antitoxins of this kind effect cures because the union between the toxin and the cell is comparatively unstable. This is not true in cases such as tetanus, in which the toxin is so strongly combined with the cells of the nervous system and other body cells that it is with difficulty that their union is broken by the addition of antitoxin. The union here between the toxin and the body cells is so stable that exceedingly large doses of the antitoxin are required, and these rarely act with any degree of success.

This explains why tetanus antitoxin is of so little use therapeutically. It is, however, of great use as a prophylactic when the toxin is free and being produced in the body.

Ehrlich, in an accurate study of the neutralization of the toxin by the antitoxin, noted that the addition of fractional amounts of the antitoxin to the L^o of the toxin (complete neutralization of one antitoxic unit) and the injection of the resulting mixtures into guinea pigs, there was not a corresponding decrease in the degree of toxicity. The toxin, therefore, seems to be made up of various parts; a part seeming to have great affinity for the antitoxins is not really toxin and is called Protoxoids. These compose about $\frac{1}{4}$ of the amount of toxins necessary to saturate one immunity unit. After $\frac{1}{4}$ of the antitoxin is added, the mixture of antitoxin becomes less toxic for the experimental animals, down to the point where $\frac{3}{4}$ of the amount of toxin necessary to saturate one unit of antitoxin is used. This fraction is considered the true toxin. Here, again, in as much as the toxicity of the mixture does not decline, it has been demonstrated that it is due to another part of the toxic molecule which has

less avidity for the antitoxin than the toxin itself and the protoxoid. This part of the molecule is called epitoxoid, true toxoid or toxon. The toxon molecule necessary to saturate one unit of antitoxin is, therefore, made up of $\frac{1}{4}$ protoxoid, $\frac{1}{2}$ true toxin and $\frac{1}{4}$ epitoxoid, toxoid or toxon.

ANTITOXINS.

Antitoxins are employed in the form of sera which may be either liquid, dry or specificated. The immunity that is produced by the use of antitoxins is passive and lasts for a period of a few weeks only. The reason for the Passive Immunity is due to the fact that the individual receives no substances which stimulate the production of protected bodies, but the individual receives, however, those protective antibodies which have been produced in the blood of some other species. When antitoxin is injected and becomes absorbed, the neutralization of these specific toxins takes place; therefore, it may be used both as a prophylactic and therapeutic agent.

The most important antitoxins used at the present time are those of diphtheria and tetanus. Some other antisera have been extensively used with good results, while others are still in the experimental stage; i. e., anti streptococci serum, anti dysentery serum, anti hog cholera serum, anti pneumococci serum and anti tubercle serum.

Diphtheritic antitoxin.

The organism is grown on Loeffler's blood serum in the incubator at 37° C., care being taken that the culture is pure.

The pure diphtheria culture is now transferred to large flasks of beef broth and incubated at 37° C. for a period of about two weeks, during which time, the rapid growth of the organism has elaborated its specific toxin and thrown it off into the broth. The culture is now examined microscopically in order to determine the absence of contamination. A preservative, such as carbolic acid or

trikresol, is added and the culture then passed through a Burkefeld filter. The diphtheritic toxin (filtrate) is then placed in the refrigerator until wanted for use.

The horses used in the manufacture of anti diphtheritic serum are removed from the detention stable where they have been confined for several weeks, during which time they are subjected to a thorough physical examination and tested for the presence of glanders by the Mallein test. They are now admitted to the antitoxin stable and injected subcutaneously with the diphtheria toxin. The first dose injected is but a fraction of a cc., then increasingly larger doses are injected until the animal is able to receive 300 cc. or more at a single injection. The intervals between the injections and the rate of increase in the doses at any time depends upon the condition of the animal. In order that a constant process of antitoxin formation may take place in the body of the horse, and that a potent serum may be produced, the injection of the toxin should be made as rapidly as the reactions, which follow each injection, will allow.

The toxin treatment usually occupies a period of about six weeks, after which the horse is allowed to rest for about two weeks in order that all of the toxin injected may be absorbed.

By means of a sterile canula, rubber tube and glass cylinders, the animal is now bled from the jugular vein under the proper aseptic and antiseptic conditions by securing as much blood as the horse can conveniently yield. After the serum separates, usually at the end of 24 to 48 hours, the clear fluid is poured into large, sterile, glass containers. A preservative is added and the material is transferred to the laboratory, where it is filtered through a Burkefeld filter.

The serum is now submitted to tests as to potency, safety and microbial contamination.

Potency Test. Varying amounts of serum are mixed with the L + dose of diphtheria toxin and injected into

a series of guinea pigs, each weighing 250 gms.

(The L + dose of toxin is the least amount of toxin which, when mixed with one unit of standard antitoxin and injected into a guinea pig of 250 gm. weight, is sufficient to kill the animal in four days.

By this test it is possible to determine the smallest amount of antitoxin which will protect the guinea pig of 250 gm. weight when the animal has received simultaneously the L + dose of toxin. This minimum amount of antitoxin represents one unit. If one five hundredth cc. of the antitoxin represents the smallest amount which is capable of neutralizing the L + dose of toxin, the antitoxin then possesses a potency of 500 units per cc.)

Safety Test. Several guinea pigs are each injected subcutaneously with 2 cc. of the serum and held under close observation until satisfied that the serum contains no injurious properties.

Microbial contamination. Inoculate culture media with large amounts of antitoxin under aerobic and anaerobic conditions. Place in the incubator, and if after a period of 72 hours no growth occurs, the serum is ready for use. If, however, a growth appears, the serum is refiltered and re-examined for microbial contamination.

The serum is now put up in sterile glass cylinders so constructed that sterilized needles and pistons may be applied and the antitoxin injected directly from the containers. Each container bears a label indicating the number of antitoxin units enclosed and the date of preparation. A number of these packages are now opened and examined for contamination, and, if free from this, the serum is ready for distribution.

Tetanus Antitoxin.

The preparation of Tetanus Antitoxin differs but little from that of the diphtheritic antitoxin. A pure culture of bacillus tetani is inoculated into large flasks of glucose bouillon and placed under anaerobic conditions (see anaerobic cultivations), or, before inoculation, drive off the free

oxygen by boiling the glucose bouillon and then covering the liquid medium by a layer of oil. Incubate these cultures at 37° C. for several weeks, after which examine microscopically; add a preservative and pass the culture through a Burkefeld filter and then through Pasteur filter.

The filtration process had better be carried out in an isolated room used only for the preparation of tetanus toxin on account of the danger of contaminating any other material or biological products with the tetanus bacillus. The tetanus antitoxin is obtained by injecting horses with the toxin along the lines laid down in the preparation of diphtheritic antitoxin. The serum is tested relative to potency, safety and absence from microbial contamination.

The unit of tetanus antitoxin is ten times the least quantity of antitetanic serum necessary to save the life of a 300 gm. guinea pig for 96 hours, against the official dose of a standard toxin furnished by the hygienic laboratory of the Public Health and Marine Hospital Service.

Anti Streptococci Serum. Bouillon cultures of the streptococcus pyogenes are killed by heating and injected into horses in increasingly larger doses. Generally but one strain of the organism is used and the serum is called "Monovalent." Frequently, however, several strains of the organism are used and the serum is then designated "Polyvalent." The procuring of the serum, etc., is carried out along the lines laid down in the preparation of the anti diphtheritic serum. The obtained antitoxin is tested in regard to safety and freedom from microbial contamination, but not as to potency, in as much as there are no known methods of standardizing the product.

Anti Gonococcus Serum. Killed cultures of *M. Gonorrhoea* are injected intraperitoneally into large, healthy rams in increasingly larger doses; finally live cultures are injected. The degree of acquired immunity is determined by frequent agglutination tests. The serum is tested as to safety and freedom from microbial

contamination but not as to potency. **Anti Dysentery Serum.** Both Monovalent and Polyvalent antitoxic sera for epidemic dysentery have been prepared by Shiga by injecting horses with the filtrate from bouillon cultures of the bacillus. It is still in the experimental stage.

VACCINES.

Preventative medicine depends to a considerable extent upon the use of vaccines, antitoxins and certain other specific biological preparations, such as diphtheritic antitoxin, small-pox vaccines, tuberculins, etc. As stated in other parts of the book, the infection of the animal organism is due to the absence of natural or acquired resistance.

An acquired resistance or immunity may, therefore, be brought about by the application of a vaccine or an antitoxin. The application of small-pox vaccine (although believed not to be bacterial in origin but will illustrate the point in question) causes a reaction in the body, or a mild form of the disease, and brings about an active immunity which is relatively permanent in duration. The use of diphtheritic antitoxins causes a passive immunity and affords temporary protection by neutralizing the diphtheritic toxin molecules.

Vaccines are weakened or modified viruses. Small-pox, black-leg and anthrax vaccines may be used with safety only on individuals free from the specific disease in question, because if given to an individual suffering from the specific disease, the introduction of the attenuated organisms or virus, would tend to increase the infection. The general action of these vaccines is, therefore, preventative, or prophylactic, and not curative.

There are several methods employed in the preparation of vaccines. The general plan is to attenuate or modify the viruses so that they may be injected into the normal animal body without danger of producing serious diseased lesions. (See active immunization).

The following methods are usually used:—

- (1) Attenuation by growth at a temperature above the optimum (see anthrax vaccine).
- (2) Attenuation by passage of virus through some species other than the animals for which the virus is specific (see small-pox vaccine).
- (3) Attenuation of virus by drying at constant temperature (see Rabbi's treatment).
- (4) Attenuation by chemical. Pathogenic bacteria are grown in the presence of weak antiseptics, which weakens their disease-producing powers.
- (5) By the simultaneous injection of a virus together with its protective serum (hog cholera).
- (6) By the combination of pathogenic bacteria with bacteria of other species antagonistic to them, as illustrated by the restraining action of yeast upon pyogenic bacteria and antagonism of the *Ps. pyocyanea* toward the bacterium anthracis.
- (7) The filtration of liquid cultures of pathogenic organism and the separation of the organism from the toxin (the toxin is used to immunize animals in the production of antitoxin).
- (8) The destruction of young living cultures of specific bacteria by moist heat at a temperature slightly above their thermol death point.

Anthrax Vaccine. (Pasteur's Method).

Cultivate the bacterium from the blood of a typical case of anthrax or agar broth.

Prepare two vaccines as follows:—

Vaccine No. 1. (less active). Grow the anthrax organisms at a temperature of 42° C. for a period of 15 to 20 days (this produces an asporogenous race). At the end of this time, suspend cultures in a sterile physiological salt solution.

Vaccine No. 2. Same as No. 1, except that it is grown for 10 to 15 days. Both vaccines must now be tested for activity and safety by animal

inoculation. No. 1 should kill white mice, but should not cause death in guinea pigs or rabbits. No. 2 should kill white mice and guinea pigs but not rabbits.

Healthy animals are injected subcutaneously with 1 cc. of No. 1. From several days to a few weeks after the injection of No. 1, the second vaccine is injected. A severe reaction with occasional death follows the use of the vaccine. These accidents can be attributed to careless methods in standardizing and administering the vaccine. The objection to this method lies in the danger of using a living organism. Good results have been obtained from the use of killed and dried anthrax organisms.

Small-pox Vaccine. The first method employed in small-pox vaccinations was inoculating healthy individuals with the virus from a mild case of the disease. Since 1796 small-pox vaccination has been carried out by vaccinating with small-pox virus. As yet, it has not been conclusively determined that the cow-pox of cattle and the small-pox of man possess intimately related causative factors, but abundant evidence proves the efficacy of cow-pox virus as a specific prophylactic against small-pox in man.

Under sterile conditions, the virus, or seed, is secured by removing the extradiate from the vesicles on infected heifers. This virus is now inoculated into calves or yearlings that have been placed in detention stables where they are inspected and carefully tested for tuberculosis. Before their admittance to the vaccine laboratory, they have passed as healthy in every way and have had their bodies scrubbed with soap and water and a weak antiseptic solution.

The virus is inoculated as follows:—

The ventral surface of the body is shaved and cleansed, and under sterile conditions, the skin is scarified in parallel lines over the greater portion of the abdomen. The stock virus is inoculated clear through the scarified area. The animal is then placed in the propagating room

and all possible precautions taken to avoid contamination by bacteria. In from 5 to 7 days, characteristic vesicles appear on the inoculated area, filled with a thick, heavy extradite. Animal removed to the operating table, the field is washed with sterile water and the contents of the vesicles are removed with a sterile pipette. (By recent order of the Federal Government, animals used in this work must be slaughtered before the vaccine is obtained and then carefully autopsied). The cow-pox extradite is mixed with about 50% glycerine, which adds as a preservative. Safety tests are made by inoculation of small portions into guinea pigs. It is then placed in the refrigerator. The glycerine and low temperature gradually destroy extraneous contamination. Potency tests are made on calves and rabbits or guinea pigs. Inoculations are made in the culture media to exclude both aerobic and anaerobic bacteria.

For the presence of the tetanus bacillus 1 cc. of the product is transferred to glucose beef bouillon and placed under anaerobic conditions at a temperature of $37\frac{1}{2}^{\circ}$ C. for about 10 days. Any growth is removed by filtration and the filtrate is injected in the guinea pig. Absence of symptoms in the injected animals shows the absence of tetanus toxin in the cultures. At the completion of these tests, the product is placed in small capillary tubes or upon ivory points, sealed in glass containers.

If kept in a cool, dark place, it will retain its activity for a period of about 3 months.

Rabies Vaccine. Originated by Pasteur in 1885, with slight modifications it continues to be the only specific treatment for rabies.

Method. A dog suffering from rabies is killed, a small portion of the brain removed, emulsified in sterile water or salt solution; inject a few drops of the emulsion subdurally into a rabbit. This inoculation should produce symptoms of "dumb rabies" and

cause the death of the rabbit in 14 to 18 days. The virulence of the strain of rabbit material is increased by making subdural inoculations until the incubation is shortened to about six days. When the rabbit shows symptoms on the sixth or seventh day after the inoculation, the virulence of the virus is called fixed virus and is now used for the preparation of the vaccine.

The spinal cord of the rabbit dying within seven days is removed antiseptically and suspended over caustic potash and dried at a temperature of 23° C. for a period of from 1 to 15 days. The patient is now vaccinated with a suspension of a spinal cord which has been attenuated by drying for 14 or 15 days. On the succeeding days of the treatment, the injection is made of spinal cord which has been less and less attenuated.

Treatment usually lasts about 21 days, or until the patient has received an injection of the least attenuated virus. It is very important that the treatment be begun as early as possible, when bitten by a rabid animal, in secured before the expiration of the incubation period.

Hog Cholera Vaccine. (Dorset-Niles Serum). Obtain the hog cholera virus by withdrawing the blood from the carotid artery suffering from the disease. Test it for activity, as a given strain of virus may not produce the acute form of hog cholera. Raise the virulence, if necessary, by passing through a series of young pigs until it uniformly produces symptoms in 4 to 6 days and death is less than 15 days. This degree of virulence is necessary in manufacturing the serum.

The blood used in the process of hyperimmunization should be obtained from susceptible pigs, weighing from 50 to 100 pounds each. The animals used as hyperimmunes should be healthy and weigh from 100 to 300 pounds and possess either natural or acquired immunity to the disease. The blood is secured from the diseased pig by allowing the blood to flow from the jugular vein into a sterile pan, or by

drawing it under aseptic conditions from the carotid artery. The blood obtained is defibrinated and the serum is secured.

The immune hogs are hyperimmunized by the slow or by the quick method.

The slow method. The animals receive several injections at intervals of every few days, each succeeding dose of the virus being increased in proportion to the weight of the animal.

The quick method. Animal is injected with one large dose of the virus, the amount determined by the weight of the animal.

In from one to two weeks after the hyperimmune animal has received the last injection of the virus, the end of the tail is severed with a sharp instrument and several hundred cc. of blood collected defibrinated, a preservative added and placed in the refrigerator. Repeat this process several times at 7 to 10 day intervals, when the animal is ready for rehyperimmunizing.

In rehyperimmunizing the animal about $\frac{1}{2}$ of the quantity of the virus used in the first process is injected. Rehyperimmunize 2 or 3 times, then relieve the animal of all its blood. Mix the different lots of serum representing the different bleedings and test the potency of the product by injecting subcutaneously four susceptible pigs weighing about 50 pounds with 2 cc. of the virus. Two of these pigs are simultaneously injected with about 20 cc. of the serum. If the serum possesses the necessary activity, the two test pigs will remain normal, except for a thermol reaction and slight, clinical symptoms, while the two controlled pigs should show symptoms in 5 or 6 days and die in less than 15 days.

The treatment now consists in simultaneously injecting hyperimmune serum and virus intramuscularly into healthy hogs. The amount of hyperimmune serum injected varies from 10 to 70 cc., depending upon the weight of the hog.

Other Vaccines in General.

After Pasteur had shown that it was possible to produce active immunity

in animals by substituting chicken cholera, anthrax and swine plague, the thought naturally suggested itself that the same should be possible in the case of some of the organisms which are pathogenic to man. Attempts in this direction showed that it was not only impossible to protect laboratory animals against infections like typhoid fever and cholera, but man could also be protected, not only by the use of living cultures, but even with the killed organisms. The great question naturally has been how large a dose of bacterial should be injected and how frequently the injection should be made in order that one might secure sufficient protection. Pfeiffer and Kolle were the first to attempt this in a human being through a bacteriolytic content of the serum. Wright then introduced a method in which he thought that by the opsonic contents of the blood the degree of protection might be indicated. It has been shown, however, that a parallel between the size of the dose and the serum content of protective substances and the degree of immunity does not exist, and the method now employed are the outcome of actual triumph.

Preparation of Vaccines.

Typhoid Fever. The culture from which the vaccine is to be made is brought to a certain degree of virulence by passage through animals and that when grown in bouillon it should yield from one thousand to two thousand million bacilli per cc. (This procedure is not absolutely necessary). The vaccine should, however, be polyvalent; i. e., it should be made from a number of different strains. The medium for the growth is generally a 1% peptone broth. Each of the strains is inoculated into separate flasks and grown at $37\frac{1}{2}^{\circ}$ C. for 24 to 48 hours, after which they are carefully mixed and sterilized in a water bath at 60° C. (Some authorities advocate 52° C.) The flask is to remain in the hot water for 10 to 15 minutes and then removed. (Some authorities indicate an ex-

posure for 1 hour. Necessity of this questioned). The sterility of the contents is now tested by placing 1 to 10 cc., according to the amount of the material, in agar or by inoculating broth.

Determine the number of bacteria per cc. by Wright's method. Mark a capillary pipette with a glass pencil about $\frac{3}{4}$ of an inch from the end, puncture the thumb and charge the pipette with a volume of blood as indicated by the mark on the pipette. Now charge the pipette with a like volume of the bacterial emulsion and then with three volumes of a 9/10% salt solution (keeping the individual portions separate from one another by little air bubbles). The blood and bacteria are now thoroughly mixed by repeatedly blowing the contents of the capillary pipette upon the slide. Small drops of this emulsion are now mounted on a clean slide and spread out like a blood film, dried and stained with Jenner's or Hasting's stain. A small square diaphragm of paper is placed in the ocular of the microscope, the red cells and bacteria are counted in successive filed until 1000 of the red cells have been counted. The number of red cells in one cc. of blood is approximately 5,000,000, and as the red cells and the bacteria must be present in the same ratio to one another as in the original units of volume, the number of bacteria per cc. of the vaccine is ascertained according to equation: Number of red cells counted : number of bacteria counted : : 5,000,000 : X.

A more accurate method has been suggested by Hopkin based upon the concentration of bacterial culture by centrifugation and the preparation of standard emulsions from the sediment. This requires an especially constructed centrifugation tube, which is prepared by the International Instrument Company, Cambridge, Mass.

Wright recommends a first injection of 750,000.000 to 1,000,000,000 organ-

isms and double this amount for the second injection.

Fearing that the injection of a large dose of organisms may be followed by a diminution in the protective substances of the body (negative phase), owing to an interaction between the normal antibacterial substances and the bacterial antigen, the individual may be temporarily less resistant to the corresponding infection. Wright, therefore, suggests that in persons who are likely to be exposed to typhoid fever soon after the first injection, this should be smaller than usual, and that its effect is to be supplemented later by a correspondingly stronger injection.

Anti-typhoid vaccination, as indicated above, is for prophylaxis only. Various attempts, however, have been made to use it as a curative agent. Some writers have expressed themselves favorably upon this point; others condemn it strongly. At any rate, it will require a great deal of investigation before definite conclusions can be reached. It is impossible to tell when and how much we inject, or as to whether it is beneficial or harmless, unless Wright's opsonic index will prove of some value as determined by future study.

Asiatic Cholera.

Two methods of vaccination against this disease have been carried out with positive results in both methods.

Haffkine's method depends upon the use of the cholera spirillum after it has been attenuated by the growth at temperature above the optimum. Vaccines of different strengths are used.

Kolle's method depends upon the use of heated (killed) cultures of the organisms.

Bubonic Plague.

The same methods employed in the vaccination against Asiatic cholera are used in the vaccination against Bubonic Plague. Cultures of the plague bacillus, killed by heating at a temperature of 60° C. for 1 hour.

Bacterial Vaccines or Bacterins for Therapeutic Purposes.

Recent studies would tend to show that bacterins (killed bacteria) may be employed as curative agents in those infections which tend to chronicity, and in which toxins play little or no part. Wright and Douglas first advanced the theory of opsonins including the suggestion that the subcutaneous injection of a given species of bacteria, killed by heating, conferred to the blood when injected a greater opsonic activity towards the species of organisms in question.

In preparing bacterial vaccines, (based upon the opsonic theory), the specific organism is isolated, grown for 24 hours at 37° C., emulsified in sterile physiological salt solution, heated in a water bath at 60° C. for ½ hour, standardized as to number of bacteria in 1 cc., and a preservative added (making the emulsion correspond to ½% lysol and tested for its sterility as in preparation of typhoid bacteria).

The use of these bacterial vaccines has brought splendid results in the treatment of furunculosis, acne, sycosis and other infections caused by pyogenic organisms.

Two kinds of vaccine are used:—the so-called autogenous vaccines; i. e., vaccines that are derived from the individual organism which is responsible for the particular infection, and the so-called stock vaccines, which are prepared from stock cultures of the specific organisms responsible for the infection.

The question as to whether or not autogenous vaccines are imperative has created a great deal of discussion. It would seem theoretically, at least, that the probable existence of many strains of a given type of organism would make the autogenous vaccines preferable to the stock vaccines.

TUBERCULINS.

Koch's Tuberculin. (Old). An inoculation of the bovine or human bact,

tuberculosis is made into several flasks of beef bouillon to which 5% glycerine has been added. The cultures are carefully placed on the surface of the medium. After an incubation at 37° to 38° C. for a period of 6 to 10 weeks or longer, the growth that slowly spreads over the surface finally falls to the bottom (it is necessary that during the incubation, the cultures remain undisturbed and have access to plenty of air without temperature fluctuations in order that they may complete the elaboration of active tuberculinic substance). The cultures are removed from the incubator and sterilized in streaming steam. Evaporate the cultures over a water bath to 0.1 its original volume; remove the bacteria by passing the cultures through filter paper and a Burkefeld filter; add a preservative.

The active substance of a tuberculin is apparently an albuminous derivative insoluble in alcohol and is elaborated during the organism's multiplication. The product used is harmless for healthy animals, but exerts a toxic action upon those affected with tuberculosis. This tuberculin is used as a diagnostic agent, not as a prophylactic agent. Its injection into individuals affected with tuberculosis is followed in from 2 to 10 hours by a rise of temperature, which continues for a few hours then subsides.

The dose of this tuberculin for cattle is 0.25 cc. 20.7 cc. By reason of its syrupy consistency and small dose, it is usually diluted with seven parts of weak carbolic acid solution; 2 cc. of the diluted tuberculin is used as the dose for cattle. The product is tested for activity by injecting known tuberculosus animals and the activity of the product is indicated by the typical reaction which follows.

Other methods than the one described above of applying tuberculin as a diagnostic agent have been instituted by Kalmette, von Pirquet and Morrow.

Kalmette's Method consists in the instillation in the eye of one drop of a 1% solution Koch's purified or refined tuberculin (prepared by treating the original tuberculin with absolute al-

cohol, washing and drying the precipitate). A positive reaction is indicated by a congestion of the palpebral and ocular conjunctiva a few hours after its application.

Von Pirquet's Method. The patient's arm is cleansed; one drop of tuberculin (old) is placed on the skin of the cleansed area and the skin underneath the drop is scarified. Two or more areas are treated in this way. It is well to scarify another small area as a control, this area to be treated with a drop of sterile salt solution, or a solution of glycerine and dilute carbolic acid in substitution for the tuberculin.

The appearance of a reddish zone in from 12 to 24 hours under the tuberculin areas indicates a positive reaction.

Morrow's Method. An ointment is prepared from equal parts of tuberculin (old) and hydrous lanolin and vigorously rubbed on a small portion of the skin of the abdomen. A distinct granular or papular eruption at the point of application after about 24 hours indicated a positive reaction.

Koch's Tuberculin in "T. R." (tuberculin residuum) is prepared by repeated centrifugation of a suspension in water of the dried and ground organisms. The supernatant fluid "t.0." after the first centrifugalization is discarded, and the final product, consisting of the constituents of the bacteria which are insoluble in water, contains the T. R. 1 cc. of the tuberculin T. R. should contain the equivalent of 1 mg. of the dry tubercle solids.

Koch's Tuberculin "B. E." (bacillary emulsion) is composed of a suspension of crushed or thoroughly ground tubercle bacilli in 5% glycerine solution. Each cc. should contain the equivalent of 1 mg. of tubercle solids.

Koch's T. R. and B. E. are used as therapeutic agents, the B. E. being regarded most favorably by clinicians. They are administered by subcutaneous injections. The initial dose recommended by Wright is one four hundredth to six hundredth mg. The intervals between the successive treat-

ments varies from three to ten days.

MALLEIN.

Mallein is used for the diagnosis of glanders. It is prepared from cultures of the bact. mallei by particularly the same method as those employed in the preparation of tuberculin. The organism used in the preparation of Mallein should be virulent. It is inoculated into flasks of glycerine bouillon having reaction of 3 and incubated at a temperature of 37° C. for several weeks. The cultures are removed from the incubator, heated in streaming steam, passed through a Burkefeld filter, the filtrate concentrated, preserved and put up in vials ready for use.

A few hours after the injection of mallein into a horse affected with glanders, a severe local reaction and a rise of temperature usually follows. The local swelling caused by the mallein is considered by some to be as diagnostic as the rising temperature.

Metchnikoff's Phagocytic Theory.

The term "phagocyte" is given to any cell capable of incorporating bacteria and of destroying them by a process of digestion.

Phagocytic cells comprise:—

1. Microphages, polymorphonuclear leucocytes.
2. Macrophages are all other leucocytes, endothelial cells and connective tissue corpuscles having phagocytic power.

When animals are subjected to an irritant, phagocytosis occurs. The leucocytes are attracted by chemotaxis to the zone of irritation and envelop the irritating substance.

The organisms that escape from one cell are seized by others, but if their multiplication is excessive they overpower the phagocytic leucocytes and invade the blood serum. The blood serum and the body fluids are likewise bactericidal, due to the disintegration of phagocytes—**phagolysis**,—the properties of these cells being imparted to the serum. This property is due to two constitu-

ents of the plasma. The one (the specific immune body) circulates in the plasma and resists a temperature of 100° C. The other, or "cytase" (digestive ferment), derived from the disintegrated phagocytes, corresponds to Buchner's "alexins" and Ehrlich's "lysins." It is destroyed at 60° C.

AGGRESSINS.

Certain bacteria may be injected into an animal in considerable quantities without producing any effect other than the temporary local disturbance following the subcutaneous administration of the material.

Certain other bacteria, on the other hand, as the bacillus of anthrax or chicken cholera, may, if injected even in the most minute dose, give rise to a rapid fatal septicemia. Within the same species fluctuations in virulence may take place, depending upon a variety of influence, but variations in the susceptibility of the inoculated subject do not furnish a sufficient explanation for the reaction so that the explanation must be put down to the activities of the bacteria themselves.

Pathogenic bacteria differ from non pathogenic bacteria in their power to overcome the protective mechanism of the animal body, and to proliferate within it. They do this by reason of a certain definite substance which they give off in the nature of the secretion, which protects them against phagocytosis. These substances were named by Bail "aggressins." The aggressins are probably absent in test tube cultures, but can be found in the animal body in the exudates occurring about the sight of inoculation in rapidly fatal infections. Bail was able to show that fatal infections could be produced in animals by the injection of sub lethal doses of bacteria if a small quantity of aggressin was administered at the same time. He believed that the aggressin paralyzed the phagocytic and other protective agencies which made it possible for the bacteria to proliferate. He further showed that animals were

successfully immunized with aggrsins. These animals were not only immune themselves, but contained a substance in their serum which permitted the passive immunization of other untreated animals.

Bail's theory has been attacked by Wasserman. Citren. Wolfe and others. These men claim that much of the aggressive character of Bail's exudates is due to their containing liberated bacterial poisons (endotoxin).

Opsonins. Wright and Douglas demonstrated that there were present in blood serum and the body fluids certain substances that had the power of rendering bacteria susceptible to phagocytosis. These substances were termed opsonins (I prepare food for). The opsonins act chemically upon certain substances within the bacteria and sensitize them. Phagocytosis depends almost wholly on these specific opsonins which are present in many normal sera for the various bacteria. Its presence was demonstrated by washing leucocytes free from all serum, when they refused, except in rare cases, to take up bacteria. Bacteria which have been placed in contact with blood serum or body fluids and thoroughly washed, will, when placed in contact with leucocytes, be taken up by them. Opsonins may be produced in animals not containing them by the process of immunization. Opsonins are destroyed at about 60° C. for thirty minutes. They will remain active for several days at 0° but will deteriorate rapidly in the withdrawn blood if stored at a temperature of 37° C. Many opsonins have the features of agglutinins and precipitins, although they bear some points of resemblance to antitoxins and complements. They possess a haptophore group with which they combine with the bacteria, and a functional group, which sensitizes the microorganisms for phagocytosis.

Opsonins may be increased in the serum of normal or infected individuals by the injection of heated (60°) cultures of these specific etiological microorganisms. These substances are called **opsonogens** or **vaccines** (see

Bacterins) and are extensively used in the treatment of various pus infections, -due to the staphylococci, and also in tuberculosis and to a less extent in pneumonia.

Wright, in his work, makes use of the so called opsonic index in order to estimate the changes in the resistance of a patient against the given infection.

The Determination of the Opsonic Index
(in order that the concentration of opsonins in an individual may be recorded).

1. By means of Wright's capsule collect blood from the finger. Seal the capsule at both ends; allow the blood to clot; and hasten the separation of the serum by a few revolutions in the centrifuge.
2. Make a bacterial emulsion by rubbing up a few loopfuls of a 24 hour slant agar culture with a little physiological salt solution; this emulsion must be even.
3. Bleed 10 to 15 drops from the ear or finger, directly into 5 or 6 cc. of a normal saline solution containing $1\frac{1}{2}\%$ sodium citrate. Centrifugalize for 5 or 6 minutes, at the end of which time the corpuscles at the bottom of the tube will be covered by a thin, greyish pellicle consisting chiefly of leucocyte. Pipette these off with a capillary pipette (by careful, superficial, scratching movements over the surface of the buffy coat forming the greyish pellicle).

The serum, the bacterial emulsion and leucocytes having thus been prepared, the test is carried out as follows:

With a greased pencil make a mark upon a six or seven inch capillary pipette, about 2 to 3 cm. from the end, and successively draw into the pipette up to the mark, corpuscles, bacteria and serum, separating them from one another by small air bubbles. Equal quantities of each having thus been secured, they are thoroughly mixed by repeatedly drawing them in and out of the pipette upon a slide. The mixture is then

drawn into the pipette; the end is sealed; incubated at $37\frac{1}{2}^{\circ}$ for about 15 to 30 minutes.

A control, or normal serum, is prepared and treated in exactly the same way. (The normal or control serum is obtained by a "Pool" or mixture of the sera of 5 or 6 supposedly normal individuals).

After incubation, the end of the pipette is broken off, the contents are again mixed, and smears are made upon glass slides in the ordinary way, and stained with Wright's or Jenner's stain, and the number of bacteria contained in each leucocyte is counted. The contents of about 80 to 100 cells are usually counted and averages taken. This average number of bacteria in such leucocytes is spoken of as the phagocytic index. The phagocytic index of the tested serum divided by that of the normal "pool" serum, gives the opsonic index. (Suppose the leucocytes of the infected individual take up an average of five bacteria. In this case, the phagocytic index is said to be five. Again, suppose the leucocytes of the normal individual take up 15 bacteria. The phagocytic index in this case would be 15. The opsonic index of the infected individual would therefore be $0.33 +$, as the normal individual phagocytic index is taken as the denominator of a fraction and the phagocytic index of the infected individual as numerator, therefore, it would be $5/15$ or $1/3$).

The opsonic index would therefore seem a fair indication as to the resistance of the particular individual to the infecting micro-organism. By the judicious use of vaccines, the opsonic index may be raised to at least 1.0 or even more, showing that the leucocytes are actively phagocytic and opsonins increased in concentration of the blood serum. In such cases, recovery will be indicated.

The opsonic index gives a fair idea as to the resistance of an individual to an infecting micro-organism.

Virulent bacteria are not phagocytized. Virulent streptococci and pneumococci are not as easily taken up as the non virulent forms. It would seem from this that some toxic or poisonous substance produced by the bacteria is antagonistic to opsonins, or it may be that an anti opsonin is formed.

The presence of opsonins in the body fluids of an animal is not absolute proof that such animal is highly resistant to infection. The resistance depends upon the activity of the phagocytes, and in certain cases where the opsonins are high in concentration the phagocytes are not active. In certain cases the reverse is true, and here the opsonins and phagocytosins are of the utmost importance during the immunity of the individual.

LEUCOCYTIC EXTRACT.

Hiss conceived the plan of injecting into infected subjects the substances that compose the chief cells, or all the cells usually found in extradiates, in the most diffusible forms and as little changed by manipulation as possible, in order that the living leucocytes which exert the protective action against bacterial infection might be considerably reinforced as directly as possible with a further supply of the weapons that they use against the microorganisms.

Hiss also assumed that extracts from the leucocytes would be more efficacious than the living leucocytes themselves, in that if they were diffusible they would be distributed impartially to all parts of the body by the circulatory system. In this way, quick absorption would relieve the tired out leucocytes and would also protect by any toxin-neutralizing or other power they might possess, the cells of highly specialized functions.

Method of obtaining these substances (for animal experiments and treatment of human subjects). Rabbits of 1500 gms. weight or over are injected intraplurally with aleuronat (prepared by making a 3% solution of starch and meat extract broth, without heating; after the starch has gone into a thorough emulsion, 5% of powdered aleuronat is added; after thorough mixing, boil for 5 minutes; fill into sterile potato tubes in quantities of 20 cc. in each tube; sterilize in the autoclave).

10 cc. of the mixture is injected into each plural cavity, in the intercostal spaces at the level of the end of the sterum, in the anterior axillary line, taking care that the lungs are not punctured. At the end of 24 hours a copious cellular exudate will have accumulated in the plural cavities. Kill the animal with chloroform and under rigid sterility open the anterior chest wall and pipette the exudate into sterile centrifuge tubes. Centrifugalize immediately before clotting can take place; decant the supernatant fluid. Add to the leucocytic sediment about 2 cc. of sterile, distilled water, and make into an emulsion by means of a platinum spatula. Make smears, stain with Jenner's blood stain and examine for possible bacterial contaminations. It is well to test for contamination by the culture method. Now add to each tube about 20 volumes of sterile, distilled water to one volume of the sediment; set aside in the incubator for 8 hours. Test again for sterility. Store in the refrigerator, where further extraction takes place, until the extract is used. Hiss and Zinsser have injected the extract subcutaneously as treatment in cases of epidemic cerebro-spinal meningitis, in lobar pneumonia, in staphylococci infections and in erysipelas with beneficial results.

ANAPHYLAXIS OR HYPER SUSCEPTIBILITY.

When a foreign proteid is introduced into the body, after a time there will appear a specific hypersusceptibility

of the animal for this proteid. If, after a definite interval, a second injection of the same substance is given, violent symptoms of illness will be produced, and often death.

As early as 1893, Behring noticed that animals highly immunized against diphtheric toxin would occasionally show marked susceptibility to injections of small doses of the toxin.

Wolfe and Esiner believe that all cells and proteid material contain a toxic substance which is characterized by its inability to produce a neutralizing antibody when injected into animals. The first injection produces a lysin for the proteid injected, which possesses the power of liberating such poisons from the complex molecules; consequently, when a second injection is given there is a rapid liberation of the toxic fraction, and an injury to the animal results. This view has been supported experimentally by Vaughn and Wheeler, who have been able to extract from various proteids toxic substances which give rise in animals to symptoms not unlike those of typical anaphylaxis.

PATHOGENIC MICRO-ORGANISMS, THE STAPHYLOCOCCI (MICROCOCCI).

The Staphylococcus Pyogenes, Aureus and Albus. These organisms are found to cause infections, such as boils, abscesses, osteomyelitis, pyemia, etc., throughout the world. These organisms stain readily in pus with aniline dyes, and the simple sowing upon ordinary media is usually sufficient for cultures, but, if pure cultures are wished, plating should be resorted to. Man seems to be considerably more susceptible to staphylococci infections than other animals. The virulence of the organisms varies and is increased by successive passage through animals of the same species, but remains unaltered for animals of other species. Virulent cultures, injected into the peritoneal cavity of animals, may kill in from 48 hours to a week, or longer, with abscess formation,

especially in the kidney. Malignant or ulcerative endocarditis has been experimentally produced; likewise, osteomyelitis. Simply rubbing the virulent cultures in the skin of man often produces furuncles.

Immunization can be secured by injections of the dead or live cocci in graduated doses. The serum possesses slight bactericidal and agglutinated properties, also a high degree of opsonic power. The serum is protective only when used slightly before or along with the injection of the organism, hence of little value. Active immunization is extensively practiced with autogenous strains of the organisms.

The variety aureus is spherical in shape. On solid media it is found singly, in pairs or in rows of three or four, but generally in irregular groups, like bunches of grapes. In liquid media, the single and paired forms are most frequent. It is gram positive. Temperature range of growth is 10° to 43° . Optimum about 30° . Grows readily on all culture media of a slightly alkaline reaction. On agar, after 24 hours, small, round, greyish white or yellow colonies appear. The characteristic orange yellow pigment may not appear until later. In broth, growth is rapid with diffused clouding, with a thin pellicle and a heavy sediment after several days. In gelatine, colonies sink into cups of liquefaction. Liquefaction is due to a thermolabel ferment substance called, Gelatinis. Milk is coagulated in three or four days time. Potato, abundant growth not as moist or smooth as on agar. Acid but no gas is produced in dextrose, lactose and saccharose media. Presence of fatty acids produces characteristic odor of cultures. Pigment appears in the aerobic but not in the anaerobic culture. The pigment is insoluble in water but soluble in alcohol, chloroform, ether and benzol. The toxins are largely intracellular. In the more virulent strains grown in moderately alkaline broth, a thermolabel hemolytic substance can be obtained by filtration through porcelain filters. Another toxic substance is

found that causes the death of leucocytes (**leucocidia**). This toxin is less stable than the one mentioned above (**staphylo-haemolysin**).

Staphylococci are more resistant than are the other non spore-bearing bacteria. An hour or more at 60° is necessary to kill watery suspensions; 70° is necessary to kill in 10 minutes. Resistance is much greater if organic material is present. Low temperatures have little effect; thirty per cent have survived 30 minute exposure to liquid air. They resist drying and direct sunlight to a marked degree. They may be found in pulp that has been dried for several months. To the germicides, especially in the presence of organic matter, they are more resistant than other vegetative bacteria.

The variety albus. This organism differs from the *pyogenes aureus* simply in the absence of the golden color. Morphologically, culturally and pathogenically, it is identical. Its toxin and enzyme producing power is generally less than the *aureus*, otherwise, its biological relationship is so close as to be demonstrated by its agglutinations in the *aureus* immune.

The Staphylococcus, Epidermidis Albus was described by Welch and may give rise to stitch abscesses. It is merely one of the non pathogenic forms of the *staphylococcus pyogenes albus*.

The Staphylococcus Pyogenes Citreus differs from the *staphylococcus pyogenes aureus* in its bright yellow or lemon colored pigment. It may be as pyogenic, but is less often found in connection with pathological lesion.

A large number of *staphylococci* differing from those mentioned above have been observed. Few of these have any pathological significance and so far as known they have no toxin producing properties. They are frequently met with as contaminations in the course of bacteriological work.

Micrococcus Tetragenus was discovered in 1881 by Gaffney in the pus of tubercular patients. Stained smears of the pus containing the micrococcus show them in the form of tetrads larger than the *staphylococci*. They

are flattened along their adjacent surfaces and are surrounded by a thick, halo like capsule. It stains easily with the usual aniline dyes, also by Gram's. It grows on all the ordinary media. On agar, the colonies first appear as transparent spots which later become greyish white, but are always more transparent than the other staphylococci cultures.

On gelatine, growth is slow; no liquefaction. Broth, is clouded. Potato, growth is white and moist showing a tendency to confluence. Milk, coagulated. Litmus milk, acid formation.

In man, the organism is probably without any pathogenic significance when found in the sputum or saliva. In a few isolated cases, it may, however, be the cause of abscess formation. Bezancon isolated the organism from a case of meningitis and Forneaca reported a case of tetragenus septicemia.

The organism is seldom found in connection with disease, but it is often found in considerable numbers in sputum examined for pneumococci or tubercle bacilli.

Experimentally, the organism is especially pathogenic for Japanese mice. If injected subcutaneously, death occurs in three or four days. Grey mice, rats, guinea pigs and rabbits are less susceptible and show only a localized reaction at the point of inoculation.

THE STREPTOCOCCI.

The Streptococci Pyogenes grow in long chains and ferment lactose, saccharose and salicin but do not coagulate milk. This group comprises most of the streptococci which cause suppurative lesions or severe systemic infections.

Streptococcic infections are endemic among all races and under all social conditions. They are more frequently found in the human being, however, than in horses, cattle and laboratory animals. The period of incubation is probably about one to three days.

Streptococci seem to be always present on the exposed surface of the body

and are capable of causing infection should any local lowered resistance occur. The symptoms of septicemia are a rapid rise in temperature to a 105° F. or over, chills, rapid, irregular and weak pulse, respiration labored, may be vomiting, and constipation or diarrhea. Headache more or less severe, sometimes delirium. Death may occur in two or three days or within a week. Mild cases may recover.

Death from septicemia causes the body to putrefy rapidly. The glandular organs, especially the spleen, tend to be swollen and soft, and parenchymatous degenerations are found to a greater or less extent. The endothelium of the heart and vessels is blood stained, which is a characteristic feature of streptococcic septicemia. Bronchitis and broncho pneumonia are usually found.

Erysipelas is an inflammation of the skin and sometimes of the mucous membrane brought about by the streptococci. The area involved is definitely outlined. Oedema may be white marked where the skin covers loose pigment. Fever with its accompaniment is present. There may be vomiting, constipation or diarrhea, severe headaches or delirium. Death may occur without any apparent complication or death may follow meningitis, pericarditis or nephritis.

Superficial cutaneous infections are met with and, if mild, may be similar to the localized abscesses caused by staphylococci. But if severe the infection is followed by rapid spreading oedema, lymphangitis, severe systemic manifestations, a grave cellulitis, often threatening life and requiring energetic surgical interference.

The respiratory organs may be invaded leading to bronchitis, pneumonia and empyema. It may be present as a secondary infection in tuberculosis. The infection of the lung and plura frequently leads to pericardial involvement.

Streptococci may invade bones and produce a severe form of osteomyelitis. If occurring in the mastoid bone, it may lead to meningitis.

In the throat and mouth, pharyngitis may be produced together with the tonsillitis that may be easily mistaken for the diphtheria. The inflammatory throat present in scarlatina is almost always due to the streptococcus. A secondary infection of this organism following diphtheria is a frequent and serious complication.

Streptococcic throat infections have recently appeared as epidemics. Several small epidemics took place in England. Severe epidemics have appeared in this country; one in Boston, one in Chicago and another in Baltimore. Investigation traced the infection in the majority of these cases to a single milk supply. Those secondary cases occurred by contact. Complications, such as suppurative adenitis, otitis, erysipelas, peritonitis and septicemia, were frequent. A capsulated hemolytic streptococcus was found in each epidemic.

From any local process, streptococci may pass into the circulation, causing sepsis. The septicemia occurring during the puerperium is often caused by this organism. Streptococci have been found in appendiceal abscesses.

Secondary foci in the viscera may take place and lead to pyemia, if localized upon the valves of the heart, septic endocarditis results. All forms of streptococcic infection, whether acute or chronic, is followed by a high mortality. The diagnosis in these cases is usually made by means of blood cultures in plain broth or other suitable media.

The streptococci vary somewhat in size. In shape they may be rounded or oval, or with one aspect flattened when they occur in pairs. The chains formed may be long or short, and a grouping into pairs is quite frequent, even when the organism is formed into chains. The organism is non-motile and without spores. It stains with the ordinary aniline dyes, gram positive, temperature range from 15 to 45, the optimum about 37°. The organism is aerobic and facultative anaerobic. A strict anaerobic species has been said to have been isolated from feces. Culture media should be

slightly alkaline in reaction. Acid is produced which has a inhibitory action upon its growth. Acids are formed from the monosaccharides lactose, saccharose and salicin. Gas production is negative. Nitrates are reduced to nitrites in some cases. Hydrogen sulphide is produced by a group called streptococcus faecalis. No pigment except a slight brownish tinge in some gelatin cultures. It is actively hemolytic which, however, is lost on cultivation. The toxic products of the organisms have been deeply investigated without any definite facts discovered. On agar, a visible growth appears in 18 to 24 hours, as small, round, translucent colonies, with even or notched borders, center thick and margins thin. The colonies show a tendency to remain discreet. In nutrient broth, the long chain varieties produce granular deposits or small flocculi or large flakes at the bottom and along the sides of the tube, leaving the remainder of the broth clear. Certain few long chain varieties produce a uniform cloudiness. The short chain varieties generally produce a cloudiness in the medium which remains so for a number of days even though a fine, granular deposit accumulates at the bottom of the tube. The gelatin colony is the same as that of the agar. Stab cultures are at first finely granular fili-form which later become beaded and may assume a brownish color. The gelatin is not liquefied. Milk becomes strongly acid and coagulation may take place. On potato, no growth results except in some cases when there seems to be an invisible growth. Loeffler's blood serum is a favorable medium.

The streptococci will die out rapidly in cultures due to the accumulation of their own products. The organism may be found alive after several weeks or months at room temperature in pus, blood or sputum. The thermal death point is 45°. Direct sunlight will kill within a few hours.

Immunity following the recovery from natural streptococcic infections is very slight, if any, and never of a

permanent nature. Septicemias once established are generally fatal and erysipelas can recur frequently. Active immunity may be produced in rabbits, goats, horses and other domestic animals by treatment with gradually increased doses of the cultures. The bacterial substances, opsonins, agglutinins and precipitins have been demonstrated in the immune serum, which, however, shows no therapeutic success.

The Streptococcus Mitis is a saprophytic type of the mouth, showing the same culture characteristics as the streptococcic pyogenes, but grows in short chains.

The Streptococcus Anginosus is a type found frequently in scarlet fever throats and differs only from the streptococcus pyogenes in coagulating milk.

The Streptococcus Salivarius is a short chain type frequently found in the mouth, rarely pathogenic, which ferments lactose, saccharose and raffinose. It coagulates milk.

The Streptococcus Fecalis is a short chain type found normally in the intestine and is occasionally pathogenic, which ferments lactose, saccharose and mannite.

The Streptococcus Equinus is a short chain type found normally in horse dung and is never pathogenic. It ferments lactose.

The Streptococcus Mucosus. This organism was described by Howard and Perkins in 1901. It was isolated by Schottmuller from cases of parametritis, peritonitis, meningitis and phlebetis. Some have claimed it to be the cause of a variety of lesions; others describe it as a harmless organism of the normal mouth. Morphologically, it shows a tendency to produce chains. On solid media, it often appears as a diplococcus. It is capsulated and is therefore similar to the pneumococcus but does not have the typical lancet shape. The fact that it ferments inulin media and on account of its agglutinating properties, it might more accurately be placed in the group of pneumococci than in the group of streptococci.

The Poynton and Paine Streptococcus (Rheumaticus). A diplococcus isolated from eight cases of acute rheumatic fever and with which Poynton and Paine produce lesions in rabbits which they considered typical of rheumatism. The organism was recovered from the blood from the pericardial fluid or the tonsil of the patients. It was described as a minute, gram negative diplococcus growing in acid media under anaerobic conditions, but would grow under aerobic conditions. Attempts to confirm their work have met with negative results. Rosenow has, however, reported a streptococcus isolated from the joints of articular rheumatic patients and has been able to produce non-suppurative arthritis, endocarditis and pericarditis in rabbits. He describes the organism as intermediate in character between the streptococcus viridans and the streptococcus hemolytica.

DIPLOCOCCUS PNEUMONIA.

(Pneumococcus, Diplococcus lanceolatus, Micrococcus Pneumonia, Streptococcus Pneumonia).

The occurrence of a diplococcus in a large majority of cases, especially of the lobar type of pneumonia, has caused this coccus to be regarded as practically specific. About 90% of all cases of acute, lobar pneumonia is caused by the pneumococcus, the remainder being due to streptococci influenza bacillus. Friedlander's bacilli and exceptionally to other microorganisms. Lobular pneumonia is also caused by the pneumococcus with almost equal regularity. The incubation period of this organism is two or three days. The onset of the disease is marked by a chill, pain and a rise in temperature. The respirations become frequent. The fever runs between 102° and 105° F. for five to ten days and then in favorable cases terminates by a sudden drop to normal within a few hours.

The pathological findings are (first stage) congestion and oedema of the lungs, followed by (second stage) the lung becoming solid, airless and of a dark red color, the alveoli showing

microscopically, a fibrous exudate with large numbers of red cells, some leucocytes and desquamated epithelium; (third stage) the lung becomes slightly softer and of a grey color, microscopically the red cells degenerate and leucocytes are increased in number. (fourth stage) Resolution takes place by liquefaction and absorption of the contents of the alveoli) and the entrance of air.

Death occurs from toxemia or complications, such as endocarditis, meningitis, etc.

For animals, the pathogenic properties of the pneumococcus varies. Natural infection is not common. Mice and rabbits are most susceptible to artificial infection; while guinea pigs, dogs, rats and cats are more resistant. Birds are nearly immune by reason of their high temperatures. Subcutaneous or intraperitoneal injections of the virulent organism from cultures or sputum kill mice and rabbits by the development of septicemia and peritonitis. The virulence of the pneumococcus may be increased by passage through susceptible animals until an extremely small dose would kill a mouse. The virulence of the cultures obtained from man may vary considerably in their virulence for animals.

The organism appears to be a common inhabitant of the respiratory tract, acquiring virulence only under some special condition that lowers the general vitality, and gaining entrance through the respiratory mucosa, and during the disease, it is frequent to find positive blood cultures; a fact which accounts for the development of complications, as meningitis and endocarditis. The toxemia results probably from lysis of the organism and it has been shown that autolysis of cultures in salt solution gives rise to a soluble toxic portion and an insoluble toxic portion.

Immunity can be shown to exist after an attack for a short time only.

Specific therapeutic agents, such as anti pneumococci sera, vaccines of dead cultures and autolysates and leucocyte extracts have been tried with some promise of results. No one

method, however, has been applied sufficiently with success enough to warrant general adoption. In the sputum, a Gram stained specimen is sufficient to detect the diplococcus, but positive identification must be made by culture. Culture medium made rich by the addition of blood serum from man or animals is used. Inoculations are made from the blood organs or sputum. Sputum injected into white mice or rabbits will often cause a fatal septicemia, and the organisms may then be obtained in pure culture from the heart's blood. The organisms appear in pairs, as oval or lancet shape cocci, with their contiguous surface flattened and the distal ends pointed. The organism may vary from this type to spherical or short bacillary form. The organism may also appear singly or in chains of a length usually not more than about six or eight individuals. Well developed capsules envelope the single organism, the pairs or the chains. There are no spores or flagella. The organism stains with the ordinary aniline dyes and is Gram positive. The temperature range is from 25° to 37°. It is both aerobic and anaerobic and grows in a slightly alkaline media. Glycerine, nutrose and dextrose media are favorable to their growth. On agar, small, transparent, finally granular colonies appear. On a serum or ascitic fluid agar, the colonies are slightly larger and more opaque. Broth is slightly but uniformly clouded. Milk is acidified and coagulated. On potato, a growth occurs but is invisible. Fermentation with acid production takes place in the majority of carbohydrates, even inulin. On blood agar, a greenish zone appears about the growth, but no clear zone of hemolysis as appears in the growth of streptococci. The differentiation from other streptococci is sometimes difficult but the following characters are important distinguishing features:—the lanceolate shape; the capsule; fermentation of inulin; absence of hemolytic power; agglutination in anti pneumococcic serum; susceptibility to lysis by the

action of bile salts. Acid is a characteristic product and if allowed to accumulate, rapidly kills the organism. The toxic products are closely united with the cell bodies and only released when the cells are broken up. The thermal death point is 52°. Light is a very efficient germicide unless protected in thick masses of sputum. Desiccation is resisted well in the sputum or in blood of infected animals. The ordinary germicides, if used in their usual strength, will kill the organism in a few minutes.

THE MICROCOCCUS INTRACELLULARIS MENINGITIDIS.

In 1887, Weichselbaum discovered a micrococcus in the exudate of cerebrospinal meningitis and called it *Diplococcus Intracellularis Meningitidis* after obtaining it in pure culture and studying its characteristics. He succeeded also in obtaining the diplococcus from the nasal secretion of the individual sick from the disease. Albreth and Ghon (1901) demonstrated the organisms in healthy individuals. It is now believed that the organism is not infrequently present in the nasal cavities. The respiratory tract through winter and spring presents a place of infection and where an increase in the virulence of the organisms take place. Meningitis, in some cases, will follow an infection of the nasal mucous membrane but not in others. Why this is so is not yet known. Infected persons, together with the material recently soiled by the nasal secretions, are dangerous.

The organism does not show marked pathogenicity for adult animals. It is most pathogenic for mice and guinea pigs, less so for rabbits and dogs. Large, subcutaneous injections in animals cause death. Mice injected into the plural or peritoneal cavity usually become sick and die within 36 to 48 hours. In man, the most marked lesions occur at the base of the brain. The cord is also infected. The exudate formed varies from a slightly turbid, serous fluid to a thick, fibrinous consistency. In chronic cases, encephalitis and dilatation of the ventricles may take place. Oc-

casionally, secondary inflammation of the nasal cavities and their accessory sinuses, catarrhal inflammations of the middle ear, acute bronchitis and pneumonia, may take place. Elsner examined the blood during the early days of the disease in forty cases and found the organism present in ten. The diagnosis of the cerebrospinal meningitis may be made by means of lumbar puncture, allowing the spinal fluid to settle, making smears of the sediment and staining by means of a blood stain, when the organism may be demonstrated, usually inside of the leucocyte, in the form of a diplococcus of a coffee bean shape or as a tetracoccus. It bears a close resemblance to the gonococcus. It never appears within the nucleus of the polynuclear leucocyte and rarely within other cells. It may be distinguished from the other organisms frequently met with in meningitis (pneumococcus, streptococcus and staphylococcus) by its rapid decoloration by the Gram solution. In many cases there are very few diplococci present in the spinal fluid, so that a failure to find them by microscopic examination should not be taken to prove that the disease did not exist, therefore, cultures from the fluid should be made immediately upon its withdrawal. The organisms tend to diminish as the disease advances. A considerable amount of fluid should be used for culture.

Immunization of animals by repeated inoculation results in the formation of agglutinins. A considerable percentage of cultures are relatively in-agglutinable. Strains that do agglutinate respond to the agglutinins developed in an animal immunized with a true strain. During recent years attempts have been made to treat the disease by injections subcutaneous and intraspinal of a meningococcus immune serum. Wasserman in 1907, obtained recoveries of 32.7% in 102 patients treated by the serum obtained from horses immunized with pure cultures of the meningococcus and toxic meningococcic extracts. Flexner and Jobling have more recently treated

the disease by the use of a similar serum injected intraspinaly, after some of the spinal fluid had been withdrawn, with excellent results. Hiss and Zinsser claim to have favorably influenced the course of the disease by the use of subcutaneous injections of leucocytic extract.

Culturally, the organism grows between 25° and 40° C., best at 37½°. Sometimes it may grow at 23°C. in artificial media. While it often lives for weeks, it may die within a few days and must therefore be transplanted to fresh material at least every two days. It grows scarcely at all in bouillon. On agar, usually a scanty growth appears. Sometimes a few colonies may grow vigorously. Comparatively good growth takes place on Loeffler's blood serum, blood serum or ascitic fluid agar. Glucose added to the media in proportion of 1% favors the growth. If the organism has been grown successfully for some time, it will produce a good growth at the end of 48 hours on nutrient agar or glucose agar. The colonies appear as a flat layer about one-eighth of an inch in diameter. They are greyish white in color, finely granular and nonconfluent unless very close together. On Loeffler's blood serum, the colonies are round, whitish, shining, viscid, with smooth and sharply defined outline. They tend to become confluent but do not liquefy the serum. The organisms are readily killed by heat, disinfectant, sunlight and drying. In the dried state, a few cocci may live for one to three days. After the cultures have been maintained for several weeks, by daily replanting, transplantation once a month will suffice.

GONOCOCCUS (DIPLOCOCCUS GONORRHOEA.)

The *Gonococcus* was discovered by Neisser (1879) in the purulent secretion of acute urethritis and vaginitis, also in the acute conjunctivitis of the new born. Bumm succeeded, in 1885, in cultivating this organism upon human blood serum. He isolated the organism in pure culture and suc-

ceeded in producing a disease by inoculating these cultures upon the healthy urethra.

The organism is usually seen in diplococci form, flattened along the surfaces, facing each other, which gives it a coffee bean shape. Stained in gonorrheal pus from acute cases, the organisms are found both intra and extracellularly; a great number of them are characteristically crowded within the leucocyte. They are never found within the nucleus. The intracellular position, which is considered diagnostic, is not found to any great extent in the secretions from chronic cases. It stains easily with the usual aniline dyes. It is decolorized by Gram, which is of differential value if applied to the pus from the male urethra. In exudates from the vagina or from the eye, the morphological characteristics are not so reliable on account of the presence of other Gram negative organisms. In the examination of a chronic discharge for the presence of the organism, it is necessary to attempt cultures by reason of the fact that a negative morphological examination cannot be regarded as conclusive.

A true gonorrheal urethritis has not been produced experimentally in animals. The infection occurs spontaneously in man. The common seat of infection is in the male and female genital tracts, in the conjunctiva, and it may also produce a cystitis, a prostatitis and stomatitis. Sometimes it enters the blood stream, giving rise to septicemia and secondarily produces endocarditis and arthritis. The organism has been found in a few cases of periostitis and osteomyelitis.

Acute infections of the genito-urinary passages in man may be followed by a prolonged chronic infection, which may remain quiescent for years and be a source of social danger.

In female children particularly, the infection is not rare, and in institutions it may travel from bed to bed assuming epidemic characters.

Subcutaneous and intraperitoneal injections of the organism into animals would produce local necrosis and suppuration, probably due to the organ-

ism's endotoxin. This toxin has been isolated by Nikolaysen from the bacteria by extracting with distilled water or a solution of sodium hydrate. The toxin will resist a temperature of a 120° and is fully as toxic for animals as the living cultures.

Christmas asserts to have demonstrated a true, soluble toxin which is denied by Wassermann and Nikolaysen, who do not believe that a general immunity is developed in individuals infected with the organism. Christmas and Torey report successful immunisation of animals, and Torey has successfully treated human cases by injections of human serum from immunized animals. Bacterins have been used with apparently real benefit in inflammations of joints and in very localized chronic infections of the urethra and bladder.

The gonococcus grows best at blood temperature. Its temperature range is from 25° to 40° . It may be grown upon nutrient agar that has been streaked with human blood. It may be grown on a nutrient agar containing 5% of glycerine. (See also media for the study of the gonococcus). After protracted cultivation, the organism will frequently grow on media containing no serum. Some strains will even grow on plain nutrient agar.

The cultures frequently die if kept at room temperature for from 48 to 72 hours. In the ice-box they may live for several weeks, and on plain nutrient agar they frequently live for one week at a temperature of 36° C.

At the end of 24 hour cultivations, a delicate growth appears, the colony is translucent, finely granular with a scalloped margin, which is sometimes scarcely to be differentiated from the culture media. The color is usually greyish white with a tinge of yellow. A streaked culture appears as a grey white translucent growth with rather thick edges.

The organism has but little resisting powers against outside influences. Weak disinfecting solutions kill it readily. It does not survive exposure to a temperature of 45° C. for six hours or a temperature of 60° for thirty minutes. Gonorrhea pus is not

very resistant to desiccation if in thin layers, but if smeared in thick layers, as on linen, it has lived for 49 days and it has also lived when dried on glass for 29 days.

MICROCOCCUS CATARRHALIS.

This organism is occasionally found in the secretions of normal mucous membrane, generally of the respiratory tract, and may be very abundant in certain diseased conditions of the mucous membrane. At times they may produce catarrhal inflammations, also pneumonia. They occur in pairs, sometimes in fours, never in chains. They are coffee bean in shape, slightly larger than the gonococcus and negative to Gram stain. According to Ghon and Pfeiffer they are of slight pathogenic significance and are of import, aside from their production of catarrhal inflammation, only in similarity to the meningococcus and the gonococcus. Certain cultures of the micrococcus catarrhalis may prove as pathogenic for white mice, guinea pigs and rabbits as the meningococcus, while other cultures are less pathogenic. The range of temperature for growth is from 20° to 40°; optimum, 37°. On nutrient agar, the growth appears as greyish white or yellowish white circular colonies of about the same size as the meningococci. The borders of the colony are irregular and abrupt. On serum agar, the growth is luxuriant. Gelatin is not liquefied. Bouillon is clouded with a frequent development of a pellicle. Milk, not changed. No gas production. It is differentiated from the gonococcus in that it grows easily on simple culture media which is not true of the gonococcus.

It is differentiated from the meningococcus by cultural characteristics and agglutination reaction. The micrococcus catarrhalis develops at temperatures below 20° C. while the meningococcus does not develop at a temperature below 25° C.

Infections due to the micrococcus catarrhalis has been successfully treated by bacterins.

Pseudo Meningococcus.

This organism was described by Elser and Huntoon as a diplococcus very similar to the meningococcus, and cannot be differentiated from it except by serum reaction. It is gram negative.

Micrococcus Pharyngis Siccus.

This organism was described by von Lingelsheim as an organism similar to the micrococcus catarrhalis from which it may be differentiated by fermentation tests, and from the meningococcus and other gram negative cocci by the firm adherence and dryness of its colonies.

Diplococcus Mucosus.

This organism was described by von Lingelsheim. It is similar to the meningococcus in its colony formation but more sticky and mucoid. It possesses a distinct capsule, which can be demonstrated by a capsule stain.

Chromogenic Gram-negative Cocci.

A study of these organisms has been made by Elser and Huntoon. They produce a greenish yellow pigment on all the ordinary culture media. At times the pigment is absent, particularly when grown upon sugar free culture media, and are to be distinguished from the meningococcus only by sugar fermentations and serum reaction.

MICROCOCCUS MELITENSIS.**Malta Fever.**

The Micrococcus Melitensis was discovered by Bruce in 1887 in the spleen in a case of Malta fever, and subsequent investigation proved it to be the causative agent of Malta fever. This disease is endemic along the shores of the Mediterranean, in South Africa, India, China, Japan, the West Indies and the Philippines. The disease does not seem to be transmitted from person to person. The period of incubation is usually about 6 to 10 days. The ordinary variety of the fever is intermittent in character and lasts for a period of from one to three weeks with intermissions and remissions, and may occur from time to time during a period of many months,

accompanied by constipation and general debilities, with various complications, such as neuralgia, arthritis, orchitis, etc. Malignant cases have been described, which may be fatal in a week or ten days. The mortality is 2% and at autopsy the spleen is found to be large and very soft. The liver is large and congested, both organs having undergone parenchymatous degeneration. The organisms are abundant in the blood and all the organs. Animals eliminate the organism in the urine, and milk of goats has been found to be a prolific source of infection in that the organisms are passed with the feces and so contaminate the milk. Safeguarding the milk will largely eliminate the disease.

The organism is an oval coccus, sometimes described as a bacillus, occurring in pairs, in irregular groups and in short chains. It is generally considered non motile but recently it has been described as motile and possesses a single flagellum at the extremity of the long diameter of the oval coccus. It stains by ordinary aniline dyes and is Gram negative. It grows at room temperature, best at body temperature, either in an acid or alkaline medium. The most favorable media for blood cultures is peptone broth to which bile has been added. On agar, after 48 hours, small, whitish or yellowish colonies appear. In broth, a slight cloudiness appears after 46 days. The culture remains alive for several weeks or months. The gelatine growth is very slow with no liquefaction.

Injected animals produce specific agglutinins, which are of practical aid in diagnosis.

Micrococcus Zymogenes.

The *Micrococcus Zymogenes* was obtained by McCallum and Hastings from a case of acute endocarditis. It has been found in a few other pathological processes. The organisms occur in pairs and sometimes in short chains. It grows on agar and ferments lactose and glucose. Gelatine is slowly liquefied.

PATHOGENIC MICRO-ORGANISMS.**Bacillus Pyocyaneus.****(Bacillus of Green and of Blue Pus.)**

The blue and green pus frequently found in many suppurating wounds is due to the action of the bacillus pyocyaneus. Gessard, in 1882, demonstrated this chromogenic microörganism as a causative factor in this peculiar type of suppuration.

The organism is usually found as a short straight rod, occasionally slightly curved. The size is subject to considerable variation. They are frequently united in pairs or in chains of 4 to 6 elements, occasionally growing into long filaments and twisted spirals. Spores are not found. The bacillus is actively motile and each possesses a single flagellum placed at one end. It stains with the ordinary aniline dyes but not with Gram.

The bacillus is widely distributed in nature; it is found on the healthy skin of man, in the feces of many animals, in water contaminated by animal or human material, in purulent discharges and in serous wound infections.

It is most pathogenic for guinea pigs and rabbits. Subcutaneous or intraperitoneal injections of 1 cc. of the bouillon culture usually cause death in from 24 to 36 hours. When smaller quantities are injected into subcutaneous tissues, the animal usually recovers, producing only a local abscess, and it is subsequently immune against a second inoculation with a dose which would prove fatal to an unprotected animal.

In man, it is found occasionally in connection with suppurative lesions of various parts of the body; frequently as a secondary infection; sometimes as the primary cause of the infection which does not usually take place unless the individual's general condition and resistance are abnormally low. Under such conditions, it may be the cause of chronic otitis media. It has been cultivated from the stools of children suffering from diarrhea and has been found at autopsy distributed throughout the organs of children dead of gastro-enteritis. It has been cultivated from the spleen at autopsy

from a case of general sepsis following mastoid operations. Wassermann demonstrated the bacillus to be an etiological factor in an epidemic of umbilical infections in new born children.

The organism is anaerobic motile bacillus capable of growing anaerobically but under this condition produces no pigment. Grows on all artificial media at room temperature, but best at 37° C. It transmits to some of the culture media a bright green color in the presence of oxygen. On agar, a wrinkled, moist, greenish white layer is developed. The surrounding medium is bright green, which subsequently becomes darker, changing to a blue green or almost black. In bouillon, the growth appears as a delicate, fluorescent sediment, changing the color of the bouillon to green. The milk is coagulated and changed to a yellowish green color. Gelatine is liquefied. The liquefaction in stab cultures occurs first near the surface, in the form of a small funnel, and extends downward and becomes stratiform, imparting a greenish yellow color to that portion of the medium which is in contact with the air.

Two pigments are produced by this organism; the one, a fluorescent green common to many bacteria, is soluble in water but not in chloroform; the other, of a blue color (pyocynin) is soluble in chloroform and may be obtained from pure solution in long blue needles. This pigment distinguishes the bacillus pyocyaneus from the other fluorescing bacteria.

Besides the ferment that causes the liquefaction of gelatine, there is one which acts upon albumen. It is called Pyocyanase, has the power to dissolve bacteria and it is believed to have some protective power when injected into animals. By reason of this fact, it has been used as local treatment in a number of cases of diphtheria. Animal infection is followed by the production of antitoxin and antibactericidal substances. Wassermann found agglutinins present in the immune sera and Eisenberg claims that such agglutinins are active also against some of the other fluorescent bacteria.

BACILLUS PROTEUS (VULGARIS)

The *Bacillus Proteus Vulgaris* was discovered along with other species of proteus by Houser (1885), in putrefying substances. It is one of the most widely distributed putrefactive bacteria, and is usually a harmless parasite when located in the mucous membrane of the nasal cavities where it only decomposes the secretions with the production of putrefactive odor. Its pathogenic powers are usually slight. It is found occasionally in the discharge from cases of otitis media in combination with other bacteria. Houser isolated the organism from a case of purulent peritonitis, from purulent puerperal endometritis, and from a phlegmonous inflammation of the hand. Next to *bacillus coli communis* the *proteus vulgaris* appears most frequently concerned in the etiology of pyelonephritis. In this condition, together with that of cystitis, the bacillus is frequently found in pure culture or associated with other bacteria. Krogus' *urobaccillus liquefaciens septicus* was probably a variety of the proteus bacillus. Many epidemics of meat poisoning have also been attributed to members of the proteus family. Buker, from extended researches, concluded that the proteus plays an important part in the morbid symptoms which characterized cholera infantum. Levi obtained a pure culture from the vomited material and the stools in the case of a man who shortly after died of cholera morbus. The blood collected at the autopsy was sterile. Weinsbers cultivated a proteus bacillus from the putrid meat which had caused acute gastroenteritis in 63 individuals. Similar epidemics have been reported by others. In some of these the bacilli were very toxic when injected into animals but could not be recovered from the organs after death. The organism grows best at temperature at or above 25° C. on all media. It is a facultative anaerobe. The bacillus appears or occurs commonly as a broad, long rod but varies greatly in size. Flexible

filaments may be formed, which are sometimes more or less wavy or twisted like braids of hair. It does not form spores and stains readily with fuchsin or gentian violet. In broth, it produces a rapid clouding with a pellicle and mucoid sediment formation. In gelatin, the colonies are characteristically irregular with rapid liquefaction, which is, however, diminished or even inhibited under anaerobic conditions. On agar, and other solid media, the characteristic irregular colonies are produced. From a central flat, greyish white polynucleus irregular streamers grow out over the surrounding medium, giving it a stellate appearance. On potato, a dirty yellowish growth appears. Milk is coagulated with an acid reaction at first, later the casein is redissolved. In peptone solutions, endol and phenol is produced. It grows well in urine and decomposes urea into carbonate of ammonia.

BACILLUS MALLEI.

Glander Bacillus.

The *Bacillus* of Glanders was first obtained in pure culture by Loeffler and Schutz in 1882. It causes an infectious disease called glanders, which is prevalent chiefly among horses, but is occasionally transmitted to other domestic animals and man.

The organism is a rather small rod with rounded ends, usually straight, but may be slightly curved. Separate individuals in the same culture vary greatly in size and this is a characteristic of the organism. In old cultures, involutions appear as short, vacuolated almost coccoid individuals. It is stained easily with methylene blue, showing irregularity in its staining qualities; the deeply stained areas alternate with areas that are faintly stained or entirely unstained. This staining irregularity is characteristic. The organism is non motile and does not form spores. It is decolorized by Gram's.

Infection with the glander bacillus occurs spontaneously most frequently in horses. It may occur in asses, cats and more rarely in dogs. The disease, in some cases, infects man, if in

habitual contact with horses. Cattle, dogs, rats and birds are immune. Experimental inoculations have been successful in guinea pigs and rabbits.

The infection takes place through the mucosa of the mouth and nasal passages and occasionally through the digestive tract in horses. It is believed that injury to the skin or mucosa is necessary for the entrance and the development of the bacilli.

In horses, the disease occurs in an acute or chronic form, depending upon the susceptibility of the subject or the relative virulence of the organism. The acute form of the disease is usually limited to the nasal mucosa and the upper respiratory passages. It begins with fever and prostration after 2 or 3 days; there is at first a serous nasal discharge which later becomes seropurulent; coincident with this, there is ulceration of the nasal mucosa and swelling of the neighboring lymphatic glands, which may break down and form pus discharging sinuses and ulcers. The lungs now become involved and death follows within 4 to 6 weeks. The chronic type is accompanied by multiple swellings of the skin and general lymphatic enlargement, and in this form is spoken of as "farcy." In this type, the onset is more gradual, together with the nasal inflammation. The swelling of the skin in some cases shows a tendency to break down and ulcerate. The disease may last for several years and may occasionally end in a cure. This is by far the most frequent type of the disease in horses. When the disease occurs in man, it is quite like that of the horse except that the point of origin is more frequently in the skin than in the nasal mucosa. The onset of the disease is violent, with fever and systemic symptoms. A nodule appears at the point of infection surrounded by lymphangitis and swelling. Occasionally a papular eruption occurs, which may become pustular and clinically may simulate small pox. Death usually follows in 8 to 10 days. The chronic form in man is much like that in the horse, but is more often fatal.

The diagnosis of glanders may be made by isolating and indentifying the organism from the center of the glander nodule, the nasal secretions and occasionally from the blood. In the majority of cases, however, isolation is difficult and animal inoculation becomes a necessity. Intraperitoneal inoculation with material containing the bacillus is made into male guinea pigs, which leads within two or three days to tumefaction and inflammation of the testicles. This method is spoken of as "Strauss Test," which should be reinforced by cultures of the testicular pus, the spleen and the peritoneal exudate of the animal on potato.

The organism is aerobic but growths may also take place under anaerobic conditions. The temperature range is 22° to 43° C., optimum $37\frac{1}{2}^{\circ}$ C. It grows easily on all culture media, whether neutral or slightly alkaline or slightly acid in reaction. Glycerine or small quantities of glucose added to the media favors its cultivation. On agar, the colonies appear after 24 hours as yellowish white, first transparent, later opaque spots, with even border. Old cultures become more yellow. On gelatin the growth is slow, of a greyish white color, with no liquefaction. In broth, there is at first (clouding later) a heavy, slimy sediment with pellicle formation. Broth later assumes a brown color. Milk is coagulated. Litmus milk indicates acid. On potato media, which is not too acid, an abundant growth appears within 48 hours, completely covering the surface as a yellowish, transparent, slimy mass, which grows darker until it becomes a deep reddish brown. This growth is considered diagnostic, although the potato growth of the bacillus pyocyaneus is very similar.

The culture will, if kept cool and in the dark in sealed tubes, live for months and years. Sunlight, if strong, will kill it within 24 hours. Heat will kill it if exposed for 2 hours to 60° C.; one hour if 75° C. Its resistance to chemical disinfections as well as drying is not very high.

The toxin (Mallein) belongs to the class of endotoxins and is obtained by ex-

traction of dead bacilli (see Mallein under vaccines). It differs from other bacterial poisons in its extreme resistance to temperatures of a 120° C. and prolonged storage. It is not a powerful poison to healthy animals, as considerable doses can be given without producing death. It is used for diagnostic purposes. The injection of the Mallein may cause reactions in the presence of other diseases than glanders, such as bronchitis, periostitis, etc., and is therefore not so valuable specifically as tuberculin for diagnosis.

Recovery from glanders will not produce immunity. Agglutinins are formed in the serum of subjects suffering from the disease, and may be used for diagnostic purposes if used in dilutions of at least one to five hundred.

BACILLUS ANTHRACIS.

The bacillus of anthrax was first observed in the blood of infected animals by Pollender in 1849. Experimental infection in animals with the blood containing the bacilli was made by Davaine in 1863.

The bacillus causes an acute infectious disease. Is very prevalent among animals, particularly, sheep and cattle. The infection not infrequently occurs in horses, hogs and goats. It is the most wide spread of all infectious diseases. It is more common in Europe and Asia than in America. Its ravages among the cattle in Russia and Siberia, the sheep in France, Hungary, Germany, Persia and India have been more severe than those of any other animal plague. Local epidemics have occurred in England and it is there called Splenic Fever.

The disease also occurs in man, particularly among stablemen, shepherds, tanners, butchers and those who work in wool and hair. Two forms of the disease have been described; the external anthrax, or malignant pustules, and the internal anthrax of which there are intestinal and pulmonary forms, the so-called wool-sorter's disease. The bacillus is a non motile straight rod. In the blood of animals, they are usually single or in pairs. Grown on artificial media, they form

long threads. The end of the individual bacillus is square. In the threads, the corners of the bacillus are so sharp that the ends in contact in a chain often touch each other only at the corner, leaving an oval chink between the ends of the organism. On artificial media, the organism forms oval spores, centrally located, which are not found in the blood of animals. The organisms are easily stained by the usual aniline dyes and are gram positive. Especially stained organisms from the animal tissues or the blood occasionally shows a capsule. This has never been demonstrated in cultures on ordinary media.

The anthrax bacillus is pathogenic for cattle, sheep, guinea pigs rats and mice, their degree of susceptibility varying greatly, even among different members of the same species, as shown by the high resistance of Algerian sheep and the high susceptibility of the European variety. Dogs, hogs, cats, birds and cold blooded animals are relatively immune. The organism is definitely pathogenic to man though less so than for cattle, etc.

Separate races of the organism may vary much in their virulence. A single strain may remain constant as to virulence, if preserved, dried upon threads or kept in sealed tubes in dark places. The virulence of the organism may be reduced by the various attenuating procedures, which is of importance in prophylactic immunization. Experimental inoculation subcutaneously is followed at first by no symptoms, and some animals appear perfectly well until a few hours or less before death. The duration of the disease depends upon the resistance of the infected subject. The quantity of the infectious material injected has little bearing on the outcome, as a single bacillus is frequently sufficient to bring about a fatal result. The bacilli are not found in the blood until immediately before death. They are, however, conveyed from the point of inoculation by the blood and lymph streams to all the organs, as has been demonstrated when the tail or ear of animals was inoculated,

without preventing a fatal infection. The bacilli do not, as a rule, multiply in the blood, at least, not at first. They may proliferate at the point of inoculation and probably in the organs and when the resistance of the animal is overcome. They invade the circulation and multiply within it. Autopsy upon such animals shows at the point of inoculation, edematous, hemorrhagic infiltration. The spleen is congested and enlarged. The kidneys are congested and there may be hemorrhagic spots upon the mucous membrane. Death brought about by the anthrax bacillus is probably due in a large extent to obstruction of the capillaries, although a true toxin has never been demonstrated, the toxic clinical picture of the disease presented in some animals and in man precludes the possibility that such poisons do not exist, though neither the culture filtrates nor the dead bacilli have any noticeable toxic effect upon test animals and exert no immunizing action.

Infection of animals takes place by way of the alimentary canal. The spores of the bacteria resist the gastric juice and develop into the vegetative form in the intestines, where they increase and invade the system. Subcutaneous infections may occur when there are small punctures and abrasions about the mouth. When infection takes place upon a visible part, there is formed a diffused local swelling, somewhat like a large carbuncle, the center of which is marked by a black, necrotic slough, or may contain a pustular depression. Infection by way of inhalation is rare among animals. The disease in infected cattle and sheep is very acute and kills within one or two days. There is about 80% mortality. In man infection generally takes place through a small cutaneous abrasion. It may also occur by inhalation and through the alimentary tract. The cutaneous infection occurs usually through an abrasion of the skin in men who handle live stock, in butchers and tanners of hides. The primary lesion, appearing at the sight of inoculation within 12 to 24 hours,

resembles an ordinary small furuncle, with a central vesicle filled with a sero sanguineous and later a sero purulent fluid. This changes in the center to a black, necrotic mass which is surrounded by an edematous areola. If early and prompt excision is made of the mass, the patient recovers; if not, local gangrene and general systemic infection may occur and lead to death within 5 or 6 days.

The pulmonary infection (wool-sorter's disease), rare in this country, occurs in persons who handle raw wool, hides or horse hair, by their inhaling or by their swallowing the spores. The disease has manifested itself as a violent, irregular pneumonia, which, in the majority of cases, leads to death.

Infection through the alimentary canal, rare in man, usually takes place through the ingestion of uncooked, infected meat, the initial lesion locating in the small intestine, producing violent enteritis, with bloody stools and great prostration, which results in death.

The anthrax bacillus grows very luxuriantly under aerobic conditions, while it develops slowly and tersely under anaerobic conditions. Its optimum temperature is $37\frac{1}{2}^{\circ}$ C. but will grow at temperature as low as 15° and as high at 45° . It may be cultivated upon all the ordinary media; a slightly alkaline or neutral media seems to be the optimum reaction. On agar plates, vigorous colonies appear in 12 to 24 hours. They are irregular in outline, wrinkled, and if examined under the microscope, they seem to be made up of hair-like tangle of thread spreading in wavy layers from a more compact central knot. On gelatin plates, they appear within 24 to 48 hours as opaque pin head size white disks. As the colony increases in size, their outline becomes less regular, and under the microscope is similar to that of the agar plates. Liquefaction takes place in about three or four days. In the gelatin stab there is at first a thin, white line along the puncture. This growth continues into the formation of thin filaments, which diverge from

the stab and take on an appearance not unlike a small, inverted "Christmas tree." Liquefaction begins at the top. In broth there is at first a rapid growth with uneven clouding and a pellicle formation, later a slimy mass somewhat like a cotton fluff appears. On potato, there is a rapid, white, dry growth. Milk is slowly acidified and coagulated.

On account of the spores, the anthrax is extremely resistant to chemical and physical agents. The vegetative form is killed by an exposure to a temperature of 54° C. for ten minutes. The spores in a dry state will live for many years. The exposure of the spore to dry heat at 140° C., for three hours, is necessary to kill. Live steam at 100° C. kills them in from 5 to 10 minutes. Low temperature does not seem to have a great deal of effect on them. The spore's resistance to chemicals varies with different strains. Direct sunlight will kill the spore within 6 to 12 hours.

Active immunization of small laboratory animals is very difficult but can be accomplished with extremely attenuated cultures.

Passive immunity by means of serum, of actively immunized sheep has been produced and practically applied by Sobernheim. The injection of such serum has been found to protect animals from anthrax and to confer an immunity which lasts often as long as two months.

No specific nor bactericidal nor bacterialitic properties have been demonstrated in the immune serum. Agglutinins have not been satisfactorily demonstrated.

ANTHRAX-LIKE BACILLI.

In nearly all laboratories there are strains of true anthrax bacilli which have become so attenuated that they are practically non pathogenic. They do not, however, differ from the virulent organisms in their culture or morphological characteristics. There are likewise in the laboratory certain non virulent bacteria which do not resemble the anthrax bacillus culturally but do so morphologically (see below).

Bacillus Anthracoides. This is a non-pathogenic, gram positive organism, indistinguishable from the bacillus anthracis, except morphologically the ends are more rounded and culturally the growth is more rapid, together with a more rapid liquefaction of gelatin.

Bacillus Radicosus. This is a non-pathogenic organism cultivated from city water supplies. Morphologically, it is somewhat larger than the anthrax bacillus and the individual bacilli are more irregular in size. Culturally, the growth is most active at room temperature, with very rapid liquefaction of gelatin.

Bacillus Subtilis. (Hay Bacillus). This is a practically non-pathogenic Gram positive organism found in brackish waters and infusions of vegetable matter, and occasionally as a saprophyte or secondary invader in chronic suppurative lesions, as in old sinuses and infected wounds. It is not very closely related to the anthrax bacillus. It occurs as straight rods and is actively motile in young cultures in which the bacilli appear singly or in pairs. In older cultures, chains are formed and the bacilli become motionless. Spores are formed, usually slightly nearer one pole than the other. On gelatin and agar the bacilla grow as a dry, corrugated pellicle. Gelatin is liquefied. Microscopically the colonies are irregularly round with fringed edges and made up of interlacing threads.

BACILLUS DIPHTHERIA.

(Klebs-Loeffler Bacillus)

The Bacillus of Diphtheria was discovered by Klebs in 1883, having observed the bacillus morphologically from the pseudo membranes of diphtheritic throats. An organism was isolated and cultivated by Loeffler in 1884, which corresponded to the morphological characters of the bacillus discovered by Klebs. He inoculated the organisms upon the injured mucous membrane of animals and produced lesions which resembled the false membranes of the disease in human individuals. Loeffler was, however, very conservative in his de-

scription of this organism as a causative agent of diphtheria by reason of his failure to find the organism in all cases examined, and his finding the organism in the throats of a healthy individual, together with his inability to explain the systemic manifestations of the infection. All existing doubt as to the etiology of the disease was overcome by Loeffler's further studies together with the publication of articles, on the nature of the toxin produced by the diphtheria bacillus, by Roux and Yersin in 1888.

The bacillus of diphtheria is subject to a number of morphological variations which depend to a certain extent upon the age of the culture and upon the constitution of the medium of which it is grown. These factors do not, however, control the appearance of the organism with any degree of regularity, in as much as all of the variations may be observed in the same growth. The difference in its morphology probably represents stages in the growth and degeneration of the individual organism. Certain characteristics in the morphology of the organism facilitate its recognition. They appear as slender, straight or slightly curved rods. Their thickness throughout their length is rarely uniform. They may show club shaped thickenings at one or both ends. Sometimes they are more thick at the center and taper towards the ends. If they are thickened at one end, they take on a slender wedge-shaped form, and are usually straight, of smaller size than the others mentioned and stain uniformly. This type has been referred to by Beck as "ground type" and believed by him to be young individual. Branched forms have also been noted, which are probably abnormal or involution forms. The organisms stain readily with watery aniline dyes. An irregularity of staining is a characteristic of diagnostic value and is best obtained by the use of Loeffler's alkaline methylene blue, which, if applied for from 5 to 10 minutes, will cause the bacilli to be traversed by stained and unstained bands, which give to the organisms a striped or

beaded appearance. If the organisms are long, they may take on the appearance of short streptococci; others may appear granular. The bacilli of about 18 hours' culture may show stained oval bodies, most frequently situated at the end, and are spoken of as polar or Babes-Ernst bodies. Special stains for these bodies have been brought out by Neisser and others, who claim for them differential value in distinguishing the diphtheria bacillus from other morphologically like organisms. These polar bodies are probably chromatic granules. The organism is stained by Gram, but care must be taken in timing the stain. Carelessness may lead to irregular results.

The bacillus of diphtheria causes a specific local action upon mucous membranes, the so-called pseudo membrane. The disease depends in part upon the mechanical surface of this membrane, and in part upon the toxins which are produced by the organism. The most frequent sites of diphtheria are the mucous membranes of the throat, larynx and nose. Occasionally they have been found in the ear, upon the mucous membrane of the stomach and vulva and upon the conjunctiva and skin. The organism may extend from the larynx and cause a diphtheritic bronchopneumonia. Although the organism has been isolated after death from the spleen and liver, a true diphtheritic speticemia is not probable.

The organism is very pathogenic to dogs, cats, fowls, rabbits and guinea pigs. Rats and mice will resist it, if administered in extremely large doses. Membranes analogous to those found in man, have been produced in susceptible animals, but only when mechanical injury to the mucosa has preceded the inoculation with the bacillus. If small quantities (one-half to 1 cc.) of a broth culture are injected subcutaneously into a guinea pig, symptoms appear within 6 to 8 hours which are followed by death within 36 to 72 hours. At autopsy, a serosanguineous exudate will be found at the point of inoculation. The lymph glands are edematous.

The kidney, liver, spleen and lungs are congested. There may be exudates in the pleural and peritoneal cavities. A severe congestion of the suprarenal bodies is characteristic and almost pathognomonic.

Agglutinins for the diphtheria bacillus have been developed in an amount to act in one to five thousand dilutions of the serum by the injections of the bodies of the organism into animals. The serum of convalescent patients has ordinarily but little agglutinating power. The test is not used in diagnosis. Antitoxins have been prepared and used in treatment with very beneficial results (see diphtheria antitoxin). Active immunization has been recommended by Theobald Smith by the use of mixtures of toxin and antitoxin, in this way producing an immunity. There are a great number of objections to this method of immunization.

The organism is an aerobe, but will grow under anaerobic conditions in the presence of carbohydrate. The temperature range is 19° to 42° C., optimum $37\frac{1}{2}^{\circ}$ C. Any temperature above this impedes the development of the toxin. The organism is isolated from mixed cultures very readily. Cultures are taken from throats and placed upon Loeffler's blood serum, upon which they are permitted to grow at $37\frac{1}{2}^{\circ}$ C. for from 18 to 24 hours. An emulsion is now made from the growth with about 5 cc. of bouillon; two or three loopfuls of this emulsion is streaked over the surface of a sugared agar, incubated for 24 hours and the characteristic colonies transferred to Loeffler's media.

The diphtheria bacillus grows readily on most of the rich laboratory media. The most favorable reaction for its growth is probably about $\frac{1}{2}\%$ alkalinity. Loeffler's media is the most widely used media for the cultivation of the organism. Swabs from suspected throats are smeared over the surface of Loeffler media and incubated at $37\frac{1}{2}^{\circ}$ C. At the end of 12 to 24 hours minute, greyish white, bristling colonies of the diphtheria bacillus are developed. These enlarge and grow to such an extent that they

outstrip the accompanying micro-organisms. This method is of value for diagnostic purposes. On agar, the colonies appear within 24 to 36 hours as small, translucent, greyish spots, quite characteristic and easily recognized. The colonies become irregularly round or oval, with a dark nucleus like center, fringed by a loose, coarse, granular disk. The edges of the colony are irregular. The addition of 1% dextrose, 2% nutrose, 6% glycerine renders the agar favorable for rapid growth but makes it a very poor media for the preservation of the culture. A meat infusion gelatin is a favorable media but because of the low temperature at which the media must be kept, the growth is very slow. Gelatin is not liquefied. The organism grows readily in milk with no coagulation. Endol is not produced in peptone solutions.

The organism has a thermal death point of 58° C. It is killed if exposed to a boiling heat for one minute. Low temperatures are borne readily. Desiccation and exposure to light are not as fatal to it as to most other pathogenic organisms. Chemical disinfectants will kill the organisms readily.

PSEUDO DIPHTHERIA BACILLUS. (*Bacillus Hoffmanni*).

The Pseudo diphtheria Bacillus was described by Hoffmann in 1888, who cultivated the organism from the throats of normal individuals, and in several instances from the throats of diphtheritic persons. The organism resembles the diphtheria bacillus but differs from it in its non-pathogenicity to guinea pigs. It was at first regarded as an attenuated form of diphtheria bacillus, but further study showed it to be unquestionably a separate species. It is a non-motile bacillus, shorter and broader than the bacillus of diphtheria. It is usually straight and may be slightly clubbed at one end. Stained with Loeffler's methylene blue, it may show unstained bands, but unlike the diphtheria bacillus, these bands rarely number more than one, and never

more than two. No polar bodies have been demonstrated by special stains. Distinguished culturally from the diphtheria bacillus, it grows more luxuriantly upon simple media. On agar plates, the colonies are larger, less transparent and more white. In liquid media, there is clouding and less tendency to pellicle formation. It does not form acid upon the various sugared media. Animals immunized with it do not possess increased resistance to the diphtheria bacillus. It is entirely innocuous to ordinary laboratory animals.

BACILLUS XEROSIS.

The *Bacillus Xerosis*, almost indistinguishable with the diphtheria bacillus, was discovered by Kutschert and Neisser from the eyes of patients suffering from a chronic conjunctivitis called Xerosis. They believed it to be the etiological factor in this disease, but the fact that it has been frequently isolated from normal eyes, precluded it as a causative factor. It is probably a harmless parasite, found more often in the slightly inflamed than in the normal conjunctiva.

No absolute differentiation morphologically can be made between the diphtheria bacillus and the *bacillus xerosis*. Polar bodies have occasionally been seen. Its growth on Loeffler's blood serum agar, glycerine agar and in broth is probably more delicate but very similar to that of *bacillus diphtheria*. It is not easily cultivated upon ordinary meat extract media. It will not grow on gelatin at room temperature and on glycerine or glucose agar; the colonies are microscopically identical with those of diphtheria. It differs, however, from the *bacillus* of diphtheria in producing acid from saccharose but not from dextrin. It is non pathogenic to animals and does not form a toxin.

BACILLUS INFLUENZA.

(Pfeiffer Bacillus).

Epidemics of influenza can be traced back to the fifteenth century. The last serious epidemic occurred in the years 1889 to 1890. Beginning in the East, traveled through Russia, became pan-

demic in Europe, invaded America and became prevalent in China, Japan, Australia and Africa. Hundreds of thousands of people were infected and the mortality was high. Since then more or less of it has been present, especially during the winter months. Many acute inflammations of the respiratory mucous membrane due to pneumococci and streptococci give symptoms similar to those produced by the bacillus of influenza, which was finally isolated by Pfeiffer in 1892 from the purulent bronchial secretions of a patient suffering from the disease and grown upon blood agar. The *Bacillus of Influenza* is an extremely small, non motile organism of irregular length, with rounded ends, rarely forming chains. The organisms do not take the ordinary aniline dyes well and are best demonstrated by staining with a 10% aqueous fuchsin for 5 to 10 minutes, or with Loeffler's alkaline methylene blue for 5 minutes. They are Gram negative. Occasionally a slight polar stain may be observed. In the smears from the bronchial secretions, the bacilli lie close together in thick, irregular clusters without definite parallelism. The fact that they very rarely form chains is considered characteristic. The organisms are found in the nasal passages and bronchial secretions of those sick from the disease. The organs affected most frequently in man are the upper respiratory passages and lungs. The disease takes the form of a broncho or lobular pneumonia. The broncho pneumonias produced by the organism do not differ essentially from those produced from other microorganisms, consequently, a bacteriological diagnosis is imperative. The infection is not infrequently followed by abscess or gangrene of the lung, and occasionally a chronic interstitial process is developed. The organisms have been found in the middle ear, the meninges, the brain and spinal cord. Although the general character of the symptoms suggests septicemia, the organism has not been found in the circulating blood. The incubation period is short, having been shown to develop

in 24 hours. The organism may remain in the bronchial secretions of convalescents or even in the secretions of normal individuals for many years. Animals are not susceptible to the infection, excepting the monkey, in which influenza-like symptoms have been produced by rubbing pure cultures upon the unbroken nasal mucosa. Rabbits inoculated intravenously suffer from severe symptoms which are probably purely toxic. The immunity produced by an attack of influenza, if any, is of very short duration.

The organism is aerobic. The isolation is not easy. Pfeiffer succeeded in growing the bacillus upon serum agar plates which had been smeared with the pus from the bronchial secretions of patients. Agar smeared with blood is a favorable media for its growth. The organism grows well symbiotically with staphylococci which seem to create a favorable environment for their development. The organism does not grow at room temperature at $37\frac{1}{2}^{\circ}$ C., and on favorable media, the colonies appear in from 18 to 24 hours as minute, colorless, transparent, discrete droplets. In order to keep the cultures alive they should be stored at room temperature and transplanted every four or five days. The organism is very sensitive to heat, desiccation and disinfectants.

INFLUENZA-LIKE BACILLI.

Pseudo Influenza Bacillus. This organism was found by Pfeiffer in the broncho-pneumonic process of children. It is slightly larger than the bacillus of influenza, non motile and Gram negative, with tendency to form threads and involution forms. It is strictly aerobic. Woolstein believes the organism to be the same as the bacillus of influenza, after having studied it both culturally and by agglutination tests. Strains of similar bacilli have been isolated from cases of pertussis.

Koch-Weeks Bacillus. This bacillus was described by Koch in 1883 and Weeks in 1887 in connection with an acute conjunctival inflammation. Morph-

ologically it resembles the bacillus of influenza, though more slender and of greater length. It grows at a temperature of $37\frac{1}{2}^{\circ}$ C. only, and can be cultivated upon serum or ascitic fluid without hemoglobin, in which respect it differs from the bacillus of influenza. It is Gram negative.

The Bacillus of Pleuro Pneumonia of Rabbits is a small Gram negative bacillus slightly larger than the bacillus of influenza and grows upon ordinary media. The organism was described by Beck.

The Bacillus Murisepticus and Bacillus Rhusiopathiae are organisms morphologically similar to the influenza group but can be easily separated from them because of their luxuriant growth on ordinary media. They are more closely related to the hemorrhagic septicemic group of organisms.

BACILLUS BORDET-GENGOU.

(Bacillus of Whooping Cough).

This organism was discovered by Bordet and Gengou in the sputum of a child ill with pertussis. It is as yet not positive as to the specificity of this organism for whooping cough. Cultivation was not successfully carried out until 1906. Since then almost pure cultures of the organism have been obtained during the early paroxysm of the disease, and for this reason it is thought likely to be a specific cause. In early cases, true influenza bacilli have often been found, and these seem to remain in the sputum of such patients for a longer period and in larger numbers than the bacillus of Bordet-Gengou. The organism though slightly larger than influenza bacillus resembles it greatly, but shows some morphological differences and less tendency to pleomorphism. It is a small ovoid bacillus found scattered in enormous numbers among the pus cells, sometimes within the cell, in the sputum early in the disease. Occasionally it resembles a micrococcus, though generally the form is constant, slightly enlarged individuals may be encountered. The poles of the cell may stain more deeply than the center. It is stained with alkaline methylene blue, dilute carbofuchsin or aqueous fuchsin.

It is decolorized by Gram. The grouping is separated, though sometimes in end to end pairs.

Inoculated into the respiratory tract of monkeys it has failed to produce the disease. One to two cc. of an extract made by emulsifying the agar growths with a little salt solution, dried in vacuo and ground in the mortar, diluted with salt solution and centrifugalized and decanted, and injected intravenously into rabbits, will kill within 24 hours. A subcutaneous inoculation will produce necrosis and ulceration without marked constitutional symptoms. Specific agglutinins obtained from immunized animals distinguishes the organism from that of the bacillus of influenza.

It is cultivated from the sputum on a medium made by adding 100 gms. of sliced potato to 200 cc. of 4% watery solution of glycerine, steamed in an autoclave; 50 cc. of this extract is mixed with 150 cc. of 6% salt solution, then 5 gms. of agar are added. It is now melted in an autoclave and filled in quantities of 2 to 3 cc. into test tubes and sterilized. To each tube is now added an equal volume of sterile defibrinated rabbit's or human blood. The substance is mixed and the tube slanted. On such a media, growth appears after 24 to 48 hours as small greyish rather thick colonies. The second generation on this media becomes rapid and luxuriant and after several generations, they resemble the growth of the typhoid bacillus. Later, it seems less dependent upon the presence of hemoglobin than does the bacillus of influenza. It will develop at as low a temperature as 5° C. but grows best at 37½° C.

BACILLUS MORAX-AXENFELD.

The Bacillus of Morax-Axenfeld was discovered by Morax in 1896 from a type of chronic catarrhal conjunctivitis, which attacked both eyes, especially in the angles of the eye and most severe at or about the caruncle. The swelling produced is not great and there is hardly ever ulceration. The condition becomes subacute and

chronic and may be diagnosed by smear preparations made from the pus, which is especially abundant during the night. The organism is a short thick diplobacillus. It may, however, appear singly or in short chains. The ends are rounded, the center slightly bulged. They are stained by the aniline dyes and decolorized by grams. Inoculation with pure cultures has produced subacute conjunctivitis in man. The production of lesions in lower animals has been unsuccessful.

The organism is cultivated only upon alkaline media containing blood or blood serum. At the end of 24 hours on Loeffler's blood serum, small areas of liquefaction are noticed, later the entire media is liquefied. Upon serum agar, delicate, greyish, droplike colonies, not unlike those of the gonococcus, are formed.

BACILLUS OF ZUR NEEDEN.

Zur Needen described a small, slightly curved, non motile diplobacillus in ulcerative conditions of the cornea, to which he attributed etiological importance. The organism is stained by the ordinary aniline dyes, though poorly at the ends and decolorized by Gram's.

Corneal ulcers are produced by inoculation of guinea pigs.

Upon agar within 24 hours, transparent, slightly fluorescent, round, raised, rather coarsely granular colonies are formed which show a tendency to confluence. Gelatin is not liquefied. Milk is coagulated. Upon potato, there appears a thick, yellowish growth. Upon dextrose media, there is acid formation but no gas. Indol is not produced in peptone.

Bacillus of Ducrey.

(Bacillus of Soft Chancre).

This bacillus was first described and obtained in pure culture by Ducrey in 1889. It produces a lesion, which occurs usually upon the genitals or the skin surrounding the genitals of an acute, inflammatory, destructive nature and called "soft chancre" or "chancroid." The lesion begins usually, as a small pustule, which soon ruptures and forms a small, round,

depressed, irregular ulcer, with undermined edges and a necrotic floor, discharging seropurulent fluid which is extremely infectious. This ulcer spreads rapidly and leads usually to lymphatic swellings in the groin, which later give rise to abscesses spoken of as "buboes." The lesion differs from the syphilitic chancre in that there is no induration.

It is an extremely small, non-motile bacillus, generally appearing in short chains and in parallel rows, though it may be found irregularly grouped. It stains easily though irregularly with aniline dyes. More stained at the poles, decolorized by Gram's. The bacilli are found in the pus, often within the leucocyte. Various investigators have succeeded in producing lesions in man by inoculating with pure cultures. Attempts to inoculate animals have been unsuccessful.

The organism grows on agar medium to which blood has been added. Coagulated blood kept for several days in sterile tubes is a very favorable medium. The organism is isolated by aspirating an unruptured bubo with a sterile hypodermic syringe and transferring the pus in quantities directly to the agar. If no buboes are present, the primary lesion may be cleansed with water or salt solution, material scraped from the bottom of the ulcer by means of a stiff platinum loop and smeared over the the surface of blood agar plates. After a period of 48 hours, small, transparent, grey, finely granular, isolated colonies appear upon the agar plate. These rarely grow larger than pin head size. The cultures die readily at room temperature but may be kept alive in the incubator for a week or more.

SPIRILLUM CHOLERAE ASIATICAE.

(The Comma Bacillus of Koch).

This organism was discovered by Koca in 1883 in the dejecta of patients suffering from Asiatic cholera.

Asiatic Cholera, a disease occurring spontaneously only in man, is endemic in eastern countries, particularly India. From time to time it

has become epidemic in Europe and Asia, not infrequently sweeping over almost the entire earth. The last great epidemic began about 1883, during which time there were 800,000 victims in Russia alone, and reached Germany in 1892. From there it entered America and Africa.

The vibrio or spirillum of cholera is a small, curved rod. The curvature may vary from the comma like form to a distinct corkscrew like spiral, with one or two turns. It is actively motile by reason of a single polar flagellum. The comma form predominates in young cultures, while the longer forms are more numerous in old cultures. Prolonged artificial cultivations without passage through the animal body tend to change the form of the organism into a straight type. They take the ordinary watery aniline dyes well and are decolorized by gram. The infection is essentially of the intestine and contracted by the injections of the organisms with water, food or contaminated material. A few organisms entering into the stomach may be checked by the normal gastric secretions by reason of the organism's sensitiveness to an acid reaction. Entering the intestine, however, they proliferate and rapidly outgrow the normal intestinal flora. Autopsies show extreme congestion of the intestinal walls, with occasional ecchymosis and localized necrosis of the mucosa, with swelling of the solitary lymph-follicles and Peyer's patches. The organisms penetrate the mucosa and lie within it in the layers next to the submucosa. The intestines are filled with watery, slightly bloody, or "rice water" stools, which is characteristic and from which pure cultures of the organism may be isolated. They are, in fact, found only in the intestines and their contents. The parenchymatous degeneration in other organs is of toxic origin. In animals the disease never appears spontaneously so that special methods were necessary in order to produce the disease experimentally. Subcutaneous inoculations, unless in large quantities of the organism, in rabbits and guinea pigs very seldom

produce more than a slight illness. Intraperitoneal inoculation, if in proper quantities, generally leads to death. Different strains of the cholera spirillum vary greatly in their virulence and this may be enhanced by repeated passage through animals.

The poisonous action of the cholera organism depends upon the formation of true secretory toxins and upon endotoxins. Which of these is paramount in producing the disease cannot be stated definitely.

Active immunisation is accomplished by inoculation of dead cultures or small doses of living bacteria. One attack confers protection against subsequent infections. Specific bacteriolysins and agglutinins are found in the serum of immunized animals, which are of great importance in making a bacteriological diagnosis of the true organism. For protective inoculation of man, see "Cholera Vaccine."

The organism is aerobic and facultative anaerobic. It grows between 22° C. and 40° C., with an optimum of $37\frac{1}{2}^{\circ}$ C. The method of isolation is accomplished by inoculating a set of gelatin plates, a set of agar plates and a set of Dunham's peptone tubes with the suspected material. If present in great quantities, they may be picked up from the plate colonies. When less numerous, they may be found in the topmost layers of the peptone broth after 8 or 10 days at $37\frac{1}{2}^{\circ}$ C., from which plate dilutions can be prepared and the colonies picked up and identified by means of cultural and agglutinative tests.

The organism grows on all the ordinary culture media of moderate alkalinity. Slight acidity will not, however, prevent growth. In gelatin plates at room temperature, yellowish grey pin head colonies appear within 24 hours. The colonies increase in size; the gelatin becomes liquefied. Magnified the colonies appear coarsely granular with irregular margins. In the gelatin stab, the liquefaction is funnel shaped. Upon agar plates, greyish opalescent colonies appear within 18 to 24 hours. These are easily differentiated, from other bacteria of the feces, by reason of their trans-

parency. Coagulated blood serum is liquefied. On potato, the growth is heavy and of a brownish color. Milk, the growth is heavy without coagulation. In broth, there is a general clouding with pellicle formations. In Dunham's peptone, indol is produced. The organism is not very resistant to drying. Boiling destroys them immediately. They are killed after an hour's exposure to a temperature of 60° C. The common disinfectants, in very weak solution, will destroy them after a short exposure. The organism frozen in ice may live for three or four days.

ORGANISMS ALLIED TO CHOLERA SPIRILLUM.

The examination of the stools of persons suffering from cholera have revealed, in a small percentage of cases, spirilla that somewhat closely resemble the true cholera organisms, and they are of bacteriological importance by reason of the difficulty which they add to the work of differentiation. Some bear only morphological resemblance, while others can be distinguished from the true cholera organism only by the serum reactions and the pathogenicity to animals.

The *Spirillum Metchnikovi* was discovered by Gamaleia in 1888, in the intestinal contents and the blood of fowls dying of an infectious disease, which prevailed in certain parts of Russia during the summer months, and which presents symptoms resembling fowl cholera. It is identical with the spirillum of cholera in its morphological and staining reactions. It possesses a single polar flagella and is actively motile. Culturally, it is similar to the cholera spirillum, except for more luxuriant growth and more rapid liquefaction of gelatin. It also gives the indol reaction in peptone media. Differentiation from the cholera spirillum is made by inoculating minute quantities subcutaneously into pigeons, producing thereby a rapidly fatal septicemia. It is more pathogenic for guinea pigs than is the cholera spirillum. There is no

lysis or agglutination by cholera immune serum.

The Spirillum of Finkler-Prior was isolated by Finkler and Prior in 1884 from the feces of patients having cholera nostras. It is like the true cholera spirillum, though somewhat longer and thicker and less uniformly curved and not so uniform in diameter, the central portion being usually wider than the pointed ends. Culturally, it resembles the cholera spirillum except that its growth is more rapid and thick upon the ordinary culture media. It does not form indol in peptone solutions nor does it give specific serum reaction with cholera immune serum.

The Spirillum Massauah was isolated by Pasquale in 1891 at Massauah from a doubtful case of cholera. In its pathogenicity, it closely resembles the spirillum Metchnikovi in that it is able to produce septicemia in pigeons. Culturally and morphologically it resembles the cholera spirillum. It possesses four flagella and does not give specific serum reaction with cholera immune serum.

The Spirillum Deneke was isolated from butter by Deneke. It greatly resembles the spirillum of Finkler-Prior. It does not produce indol in peptone media.

ACID FAST GROUP OF ORGANISMS.

This so-called "acid fast" group of organisms derives their name by the non-permeability of the ordinary stains unless exposed to them for a long time or by heating the solutions. The stain having entered the organism will retain it even when treated with alcohol and strong acids. The acid fast nature seems to depend upon the fatty substances contained within the organisms.

The Bacillus of Tuberculosis.

This bacillus was isolated by Koch in 1882 and established the etiological relationship of the bacillus to the disease by infecting guinea pigs and other animals with pure cultures of the bacillus, and producing the characteristic lesions. Previous to this the transmission of tuberculous material was accomplished by Klenke in 1843

and Willeman in 1865 and the tubercle bacillus had been demonstrated in tissue sections by Baumgarten early in 1882.

The tubercle bacillus is a slender, straight, or slightly curved rod usually rounded at the ends. The diameter may be uniform throughout, though more often they appear beaded and irregularly stained. This irregularity in staining generally appears in old cultures and may be regarded as vacuola. The bodies of the bacilli may bulge slightly in three or four places, presenting oval or round knobs which take the stain deeply and are very resistant to decolorization. A cell membrane has been described which confers resistance to the organism against drying, etc. It gives a cellulose reaction and the waxy material obtained from the culture by extraction is believed to be contained within it. Branched forms of the organism have been demonstrated by various observers, and by reason of this fact, it is probable that the tubercle bacillus is not a member of the family schizomycetes but belongs to the higher bacteria.

The bacilli do not stain easily with the ordinary aniline dyes. They must be exposed to the stains for a long time or the stain solution must be heated. After having been stained, however, they are extremely resistant to acids and decolorizations. For methods of staining see section on Staining Formulas.

Very young cultures are often not acid fast and it is not always possible to demonstrate acid fast bacilli in pus from cold abscesses, in sputum, in serous exudates and in lesions of the lymph nodes which can be shown by animal inoculation to be tuberculant. In this material, Much calls attention to Gram positive granules arranged singly in short chains or irregular clumps, which he believed to be non acid fast tubercle bacilli. There is no doubt as to the truth of his belief in that his work has repeatedly been confirmed. These Gram positive bodies, however, are not of great diagnostic value as other bacilli form granules of the same appearance.

Small rods and splinters are also found which are Gram positive and do not stain by the carbofuchsin method. Other organisms of the acid fast group, which may be difficult to differentiate from the tubercle bacillus, are the bacillus of leprosy and the smegma bacillus. By reason of the distribution of the smegma bacillus in feces, urine or even sputum, it becomes necessary to apply to suspected specimens the other stains which are devised for the differentiation of this bacillus from that of tuberculosis. Pappenheim's stain is the one most frequently employed for this purpose. Stained by Pappenheim's method the tubercle bacilli remain red; the smegma bacilli appears blue. Tubercle bacilli are Gram positive. When tubercle bacilli are present in extremely small numbers in the sputum and other material, it may be impossible to find them by direct examination, and often the only method of demonstrating them will be by animal inoculation. Methods of concentration have been devised by which the bacilli may be found when only a few are present. One method is to add hydrogen peroxide to the sputum. This dissolves the mucous and allows the solid particles to settle by centrifugation. Another method, much used today, is by the use of "antiformin," which is made up of equal parts of liquor soda chlorinated (sodium carbonate 600 parts, chlorinated lime 400 parts and distilled water 4,000 parts) and a 15% solution of caustic soda. The sputum is pored into a 10 to 15% solution of antiformin and allowed to stand for several hours. The other elements of the sputum, as the cells and bacteria, will be dissolved out, leaving only the acid fast bacteria in the residue. The tubercle bacilli are not killed by this process and after sufficient washing, they may be cultivated or can produce lesions in guinea pigs.

The organism produces in man and susceptible animals, a specific phenomenon of inflammatory foci, known as tubercles. Tuberculosis is the most common disease in man. Nägel

in a large series of autopsies found lesions of healed or active tuberculosis in a large percentage of cases. The disease is less common in the rural districts than in large towns. The pulmonary infection is the most common type in man, though tuberculous process may be found in the skin, the bones, the joints, the organs of special sense, the abdominal viscera, and peritoneum. Infection takes place by inhalation or through the skin, or through the digestive apparatus. Behring has caused a great deal of discussion by stating that he believed a large percentage of all cases of tuberculosis originated in childhood by way of the intestinal tract. He therefore brought to notice the problem of the virulence of bovine tubercle bacilli for human beings, as he assumed that the infection of children is due to the use of infected milk. From the contributions of Parke and Krumweide, it would seem that human adults are relatively insusceptible to bovine infection which may take place, but is unusual.

A more relative susceptibility is found under 16 years of age, and the danger of milk infection is without doubt great; in fact, one third of the cases arising from this source. The danger of bovine tuberculosis is greatest under 5 years of age.

The above statement would indicate that Behring's original statement cannot be upheld, though it does, without doubt, point to the great dangers of milk infections.

The tubercle bacillus (human) is pathogenic for guinea pigs; less so for rabbits and still less so for dogs. It is slightly pathogenic for cattle.

The work connected with the isolation of specific toxins has led to a chemical analysis of the organism, which shows it to consist of about 85½% of water; 20 to 26% of the residue can be extracted with ether and alcohol. This material consists of fatty acids and waxy substance (fatty acids in combination with higher alcohol). The residue, after the alcohol ether extraction, is made up chiefly of proteids, which can be extracted with dilute alkaline solutions

and consist principally of nucleo albumens, a fraction of which is suspected of being the pathogenic principal of the bacillus in that it shows high toxicity. The remainder contains cellulose, representing probably the frame work of the cell membrane and the ash which is rich in calcium and magnesia.

(For the toxins and their method of preparation see Tuberculins). Numerous attempts have been made to passively immunize tubercular subjects with the sera of immune animals. The methods most used for the production of such serum is that of Maragliano, who believes that there is a toxic albumin in the cultures of the tubercle bacilli, which is destroyed by the heating employed in the usual production of tuberculins. He therefore prepares his substance by filtering unheated cultures and precipitates the filtrates with alcohol. He now makes a watery extract of the bacillary bodies and with these two substances he immunizes horses. After 4 to 6 months of treatment, he withdraws blood from the horse and separates the serum. This serum called Maragliano's serum is extensively used in Italy. Its value is very doubtful.

Marmorex claims that the toxin produced by the bacillus of tuberculosis is dependent to a great extent upon the medium on which it is grown. He believed that the substance obtained in tuberculin was not true toxins of the bacillus and that the true toxins could only be elaborated by a younger phase of the bacillus, as it occurs within the animal body or on media composed of animal tissue. He therefore grows his cultures on a medium of leucotoxic serum and liver tissue. Such cultures he believes to contain no tuberculant. The sera produced by immunization with these cultures is supposed by him to have high curative powers.

The organism is aerobic with a temperature range of 30° to 42° C. with an optimum of 37½. The organism is not easily cultivated. Isolation from tuberculous material is materially aided by inoculation into guinea pigs.

The animals often withstand the acute infection produced by contaminating organisms and in 4 to 6 weeks die from the tuberculous infection. The bacilli may then be cultivated from the lymphnodes or other foci which contain only tubercle bacilli. Koch isolated the organism from the sputum as follows:—

The sputum is thoroughly washed in running water to free it from mucus. It is then washed in 8 or 10 changes of sterile water. The material for cultivation is taken from the center of the washed mass, if possible. Select the caseous material, which is often present in such sputum, and either inoculate culture media directly or inoculate animals as indicated above. On blood serum, at the end of 8 to 14 days, small, dry, greyish white, scaly colonies with corrugated surfaces appear. At the end of 3 to 4 weeks' cultivation these colonies joined together cover the surface of the medium as a dry, whitish, wrinkled membrane. The organism will grow well on agar slants to which 1 to 2 cc. of rabbit's blood has been added. Glycerine agar is a favorable media for its growth. Glycerine bouillon of a slightly alkaline reaction is an excellent culture medium. This medium is placed in thin layers into wide mouthed flasks and then inoculated by carefully floating flakes of the culture upon the surface. The organism will appear as a thin, opaque, floating film, which rapidly thickens into a white, wrinkled, or granular layer, spreading out in all directions and covering the entire surface of the bouillon in from 4 to 6 weeks. Later, portions of the membrane will sink to the bottom. The organism will also grow freely upon glycerine potato.

The life of cultures kept in a favorable environment (access of oxygen) is from 2 to 8 months. In sputum, they remain alive and virulent for 6 weeks, and in dried sputum for more than 2 months.

In fluid media, the organism is killed at a temperature of 60° C. in from 15 to 20 minutes, and at 80° C. in 5 minutes; at 90° C. in from 1 to

minutes. They withstand dry heat at a 100° C. for 1 hour. They are resistant to cold; 5% carbolic acid will kill the organism in a few minutes. If used as a disinfectant for sputum by reason of the fact that the mucous coat will protect the bacilli, the disinfectant should be allowed to act for 5 to 6 hours. Direct sunlight will kill the organism in a few hours.

The Bacillus of Bovine Tuberculosis.

The difference between the reaction to the infection in the bovine type of the disease and that of man was studied by Koch in his early work. He thought this difference to be due to the nature of the infected subjects. Theobal Smith, in 1898, pointed out the difference between the bacilli isolated from man and those isolated from cattle. He determined that the bovine bacilli were usually shorter than the human bacilli and they did not grow as well upon artificial media. Grown upon slightly acid glycerine bouillon, the bovine organism will reduce the acidity until a neutral or slightly alkaline reaction is reached. It differs in this respect from the human bacillus, which produces but slight reduction of the acidity during the first week of growth. After this, the acidity increases and never reaches neutrality. Marked differences have also been shown to exist in the pathogenicity of these organisms towards various animal species. Guinea pigs die more quickly and show more extensive lesions when inoculated with the bovine type than when inoculated with the human bacillus. The bovine bacillus will kill a rabbit within 2 to 5 weeks. The human bacillus produces a mild disease, which lasts frequently for 6 months, and at times fails to kill a rabbit at all.

Many attempts, with little or no success, have been made to infect cattle with the human organism. Infection of the human individual with the bacillus of the bovine type has been reported and proven by Smith, Parke, Krumweide and others.

Bacillus of Avian Tuberculosis. Koch discovered bacilli in the lesions of diseased fowl, which closely resembled the bacillus of tuberculosis. Nocard

and Roux differentiated these bacilli as a definite species. In its staining characteristics and in its morphology, it is almost identical with the human bacillus of human tuberculosis. Culturally, it differs, however, in that growth is more rapid and takes place at a temperature of 41° to 45° C. Guinea pigs are very resistant to this type, while rabbits die quickly from the avian tuberculosis. Prolonged cultivation and passage through the mammalian bodies will cause the organism to approach the mammalian type. Nocard has, on the other hand, succeeded in rendering the mammalian tubercle bacilli pathogenic for fowl by keeping them enclosed in celloidin sacs within the peritoneum of chickens for 6 months.

Tuberculosis of Cold Blooded Animals.

A bacillus resembling the bacillus of tuberculosis in its morphology and acid fastness was isolated by Dubarre and Terre from cold blooded animals, as fish, frogs, turtles and snakes. It is non-pathogenic to warm blooded animals but will kill a frog within 4 weeks. It grows at a temperature from 15° to 30° C. Attempts have been made to show a close relationship between the tubercle bacillus of cold blooded animals and that of warm blooded animals. Kuster, reviewing the work of many investigators, states that spontaneous tuberculosis may occur in fish, snakes, turtles and frogs, and that the organism which causes these diseases is specific for cold blooded animals and similar in many respects to the tubercle bacillus of warm blooded animals, but in them they do not produce progressive disease. The human, bovine and avian tubercle bacilli when inoculated into cold blooded animals can produce lesions which simulate tuberculosis and the bacilli may remain in these lesions for a long period of time without losing their pathogenicity for guinea pigs.

BACILLI RESEMBLING TUBERCLE BACILLI.

Bacillus of Timothy. This organism was isolated by Muller from timothy grass and dust in haylofts. Will grow

rapidly on agar and is of a deep red or dark yellow color.

Bacillus Butyricus (Butter Bacillus): This organism was isolated by Petri, Korn and others from milk and butter. It resembles the bacillus of tuberculosis in that it is slightly acid fast but can be differentiated culturally. It is slightly pathogenic for guinea pigs but not for man.

LUSTGARTEN'S BACILLUS.

Bacillus Smegmatis.

Lustgarten, in 1884, discovered an acid fast bacillus in syphilitic lesions which he believed to be the cause of this disease. It has since been shown that a bacillus which corresponds in its morphology but differing slightly in certain staining peculiarities to the bacillus described by Lustgarten, occurs as a harmless saprophyte in the normal smegma from the prepuce or vulva. The bacillus is a straight or curved organism resembling the tubercle bacillus in its morphology. It is found lying singly or sometimes in groups within the interior of cells having a round, oval or polygonal form and apparently somewhat swollen. It stains with almost as much difficulty as the tubercle bacillus, but is more easily decolorized. A method of differentiation was devised by Pappenheim (see stain) which depends upon the fact that prolonged treatment with alcohol and rosolic acid will decolorize the smegma bacillus but not the tubercle bacillus.

Attempts have been made, without success, to cultivate Lustgarten's bacillus on artificial media. The smegma bacillus has no pathogenic significance. Attempts to infect animals have been unsuccessful. It might be well to state here that although the so called smegma bacillus and the bacillus of Lustgarten are almost identical, the identity of the two bacilli has not been definitely established.

LEPROSY BACILLUS.

Bacillus Leprae.

The Bacillus of Leprosy was discovered by G. A. Hansen, in 1879, in the tissues of the nodular lesions of indi-

viduals suffering from leprosy. The organisms were found lying in small clumps intra and extracellularly, as well as in the serum that oozed from the tissue during its removal. The disease was present and widely distributed long before the Christian era, extending down through the Middle Ages to the present time. It is most common in India and China. It is found in Norway, Russia and Iceland; and in the United States in Louisiana, California and Minnesota.

The bacillus of leprosy is a small, slender, acid fast rod, resembling the tubercle bacillus in form, though somewhat shorter and not so frequently curved. The rods have pointed ends, and when stained have the unstained spaces frequently seen in the tubercle bacilli. The organism stains easily with the ordinary aniline dyes, also by Gram's.

Attempts to inoculate animals with leprosy have been unsuccessful. Subcutaneous inoculations of cultures into guinea pigs have produced local lesions which resemble the leprosy lesions in man. Duval states that he was able to continue the growth on later transfers. The bacilli are usually found in large numbers especially in the tubercles of the skin, in the conjunctiva and cornea, the mucous membrane of the mouth, gums and larynx, and in the interstitial processes of the nerves, testicles, liver, spleen and kidneys. The bacilli nearly always lie within the cells in the old centers of infection, are larger and often polynuclear. Some observers have claimed to have found giant cells similar to those of tuberculosis in the nodules. The hair follicles, sebaceous sweat glands, are often attached. A true caseation does not occur, but ulceration results.

In the anaesthetic form of leprosy, the bacilli are most commonly found in the nerves and less frequently in the skin. The organism may also occur in the blood, partly free and partly within the leucocyte, particularly during the febrile stage, which precedes the breaking out of the tubercles. The bacilli have also been found in the

intestines, in the lungs and in the sputum, but not in the urine.

The contagiousness of leprosy is far less than that of most other bacterial diseases. The question of direct inheritance of the disease from the mother to the unborn child has brought out a considerable difference of opinion. Many attempts have been made to infect healthy individuals with the material containing the bacilli without conclusive results. Arning, although he successfully infected a condemned criminal in the Sandwich Islands with fresh leprosy tubercles, did not produce positive evidence of the transmissibility of the disease in that way, as Swift pointed out that the man had other opportunities of becoming infected. A widespread idea before the discovery of the organism that the disease was associated with the constant eating of dried fish, or certain kind of food, has been abandoned.

The relation of leprosy to tuberculosis is evidenced by their similarity in many respects, and still more so by the fact that leprosy will react both locally and generally to injections of tuberculin in the same manner as tuberculosis, but to somewhat less extent.

Cultivation of the leprosy bacillus has not met with success.

Clegg, in 1908, reported that he had been able to cultivate an acid fast bacillus, from cases of leprosy, in symbiosis with ameba and cholera vibrio. By heating a symbiotic culture of the leprosy bacillus to 60° C. it was obtained in pure culture. From the first culture, different media were successfully inoculated. On agar, the surface colonies are small and brownish. Blood serum is liquefied after ten days; lactose is not fermented. Duval succeeded in obtaining cultures by the Clegg method, but in spite of extensive work along this line, the opinions are still divided as to the specific nature of the organisms cultivated by these two investigators.

BACILLUS OF RAT LEPROSY.

A disease occurring spontaneously among house rats in Odessa was first observed by Stefansky, characterized by subcutaneous induration, swelling of lymphnodes, falling out of the hair, emaciation and sometimes ulceration. In the diseased rats, under the skin of the abdomen or flank, a thickened area of adipose-like tissue, though more nodular and grey and less shiny than fat, may be found, within which are acid fast bacilli resembling the bacillus leprae. These organisms may also be found in the lymphnodes and sometimes in small nodules of the liver and lung.

The disease can be transmitted from rat to rat experimentally. The disease is not exactly like human leprosy clinically.

PATHOGENIC ANAEROBIC GROUP OF ORGANISMS.**BACILLUS TETANI.**

The infectious nature of lockjaw, or tetanus, was demonstrated by Carlo and Rattone, in 1884. Kitasato by anaerobic methods demonstrated the tetanus bacillus in 1889, and definitely solved the etiology of the disease. The bacillus of tetanus is found to occur in the superficial layers of the soil. The soil of cultivated and manured fields is thickly sown with this organism, probably because of its presence in the dejecta of some domestic animals.

The organism is a slender bacillus, the vegetative forms of which are slightly motile by reason of the numerous peritrichial flagella. The organism develops spores which are characteristically located at one end and give to it the diagnostic drum stick appearance. The vegetative forms occur chiefly in young cultures, which, after 24 hours' incubation, develop into the spore forms, and as the cultures grow old, the spores will supersede the vegetative form. In very old cultures only spore forms and spores are found. The organism stains easily with aniline dyes and also by Gram. The organism is extremely pathogenic, though viewed from the stand-

point of universal distribution of the bacilli in nature, the infection is infrequent. The spores of the organism introduced into the animal body, free from toxins, the disease may not be produced, by reason of their susceptibility to destruction by phagocytosis and to other protective agents before vegetative forms can develop and the toxin formed. Tetanus will, however, develop in animals if introduced into deep, lacerated wounds in which there has been considerable tissue destruction. Common pus cocci or other more harmless parasites may aid in furnishing a suitable media for the growth of the tetanus bacillus. There is an incubation period of from 5 to 7 days in acute cases to from 4 to 5 weeks in chronic ones. Guinea pigs inoculated with the organism suffer in from 1 to 3 days from a rigidity of the muscles nearest the point of infection, which rapidly spreads to other parts of the body and is followed by death in 4 or 5 days after the time of injection. Autopsies of animals or human beings, dead of tetanus, do not present marked lesions. The point of infection may be trifling in appearance. The organs show no pathological changes except a general moderate congestion. The bacilli are infrequently found at the point of infection and have rarely been demonstrated in the blood or viscera. Tizzoni and Creite have succeeded in cultivating the tetanus bacillus from the spleen and heart's blood of infected human beings. The pathogenicity of the organism is dependent upon the soluble toxin which it produces. It is one of the most powerful poisons known. Filtrates of broth cultures in quantities of 0.000.005 cc. will often prove fatal to mice of 10 gm. weight. Different species of animals show great variation in susceptibility. Human beings and horses are probably the most susceptible species in proportion to their body weight. The susceptibility of the horse calculated for grams of body weight is twelve times that of a mouse. The hen is extremely resistant, in fact 200,000 times more resistant than the mouse. The toxin injected subcutaneously

first produces spasms in the muscles nearest the point of inoculation. Intravenous inoculation usually results in a general spasm of all the muscles. The action of the tetanus toxin is upon the central nervous system. The manner in which the toxin reaches the central nervous system is by way of the motor nerve. The toxin injected into the circulation will reach all the motor nerve endings simultaneously, producing a general tetanus. The poison cannot pass directly into the central nervous system but must follow the route of nerve tracts (see also antitetanic serum).

The bacillus of tetanus is an obligative anaerobe and if cultivated strictly under these conditions, it will grow readily upon meat infusion broth which becomes clouded in 24 to 36 hours. Upon meat infusion gelatin, there is slow liquefaction. On agar, at the end of 48 hours, compact colonies not unlike subtilis colonies make their appearance. In agar stabs, the growth appears as fine, radiating processes grown from the central stab. Milk is a favorable media and is not changed. On potato, a hardly visible growth appears. The media may be rendered more favorable for growth by the addition of a 1 or 2% of glucose, maltose or sodium formate. The vegetative forms of the bacillus are not more resistant against heat or chemical agents than the vegetative forms of other microorganisms. The spores, however, will resist dry heat at 80° C. for 1 hour, live steam for about 5 minutes. They are killed by a 5% carbolic acid solution in 12 to 15 hours; 1% of bichloride of mercury in 2 to 3 hours. Direct sunlight will diminish their virulence and ultimately destroy them.

BACILLUS OF SYMPTOMATIC ANTHRAX.

An infectious disease occurring among sheep, cattle and goats, spoken of as "Quarter-evil" or "Blackleg" is often confused with true anthrax by reason of a slight similarity in clinical symptoms. It is, however, caused by a very different microorganism found widely distributed in the soil, from

which the infection is generally obtained. The bacillus of symptomatic anthrax is a spore bearing bacillus of drum stick shape, or spindle shape, and is anaerobic. It was first obtained in pure cultures by Kitasato. It is usually seen singly and never forms chains. In the vegetative form the organism is motile, but soon loses this power on account of the oxygen to which it is exposed. It shows well defined flagella and develops spores. The organism may be demonstrated by the aid of microscope in the blood without staining, if done soon after death. It stains easily by the simple aniline dyes or by Gram's.

The bacilli are pathogenic for cattle, sheep and goats. Most cases appear among cattle. Guinea pigs are very susceptible. Horses, little susceptible; dogs, cats, rabbits and birds are immune. Man appears to be absolutely immune. Infection takes place through abrasions or wounds, and depends to some extent upon the degree of virulence, which varies greatly in this organism. A soft, puffy, swelling appears at the point of entrance at from 4 to 24 hours after the inoculation. This area is found to be emphysematous, which spreads rapidly, often reaching the abdomen and chest in a day. It is accompanied with high fever and extreme general prostration. Death usually results within 3 to 4 days. At autopsy, the swollen area is found infiltrated with a thick blood tinged and foamy exudate. The subcutaneous tissues and muscles are edematous and filled with gas. The organs show parenchymatous degeneration and hemorrhagic areas. The organisms are found in enormous numbers in the area surrounding a central focus, immediately after death, but very few are found in the blood and internal organs. The unburied carcasses become bloated with gas and the organs filled with bubbles, by reason of the general distribution of the bacilli.

A soluble toxin is produced by the organism in considerable quantities in broth containing blood or albuminous animal fluids, but not to any extent in ordinary broth. The toxin is quite

resistant to heat, but deteriorates on exposure to air.

Arloing actively immunized cattle by subcutaneous inoculation with vaccines prepared from the tissue of infected animals. Two vaccines are prepared; No. 1 is made from the juice of infected meat, which is dried and heated to 100° C. for six hours. No. 2 is also made from the juice of infected meat heated to 90° C. for six hours. No. 1 is injected in quantities of 0.01 to 0.02 cc., emulsified in sterile salt solution, near the end of the animal to be protected. The same quantity of No. 2 is injected in the same way after 14 days.

Kitt prepared a vaccine from the powdered meat which was heated to 94° C. for six hours, which has been largely used in America. A passive immunization with the serum of actively immunized sheep has been used in combination with the methods of active immunization.

The organism grows readily under anaerobic conditions upon the ordinary media, which may be rendered more favorable by the addition of glucose glycerine or nutrose. The organism will grow equally well on slightly alkaline or slightly acid medium and is an active gas producer. On agar plates the colonies appear round with a compact, slightly granular center from which a thin zone is given off. This zone will under the microscope appear as a tangle of fine threads. In agar stabs, the growth appears within 18 hours and spreads into the media as a diffused fine cloud. Gas bubbles are formed which later increase to such extent as to cause extensive splitting of the medium. On gelatin plates, round or oval colonies with a compact center, about which are fine, radiating filaments, make their appearance in about 24 hours. The gelatin is liquefied. In gelatin stab cultures the growth is less rapid, but is similar to the growth in agar stabs.

BACILLUS OF MALIGNANT EDEMA. **(*Bacillus Oedematis Maligni*).**

Pasteur in 1877 described an organism which he isolated under anaerobic conditions in impure cultures from the tissues of guinea pigs and rabbits which he had inoculated with putrefying animal tissues. He named it "*Vibrio septique*." Koch, in 1881, suggested the term "*bacillus of malignant edema*," by reason of the fact that the organism did not produce a true septicemia. The organism was later found to occur in garden soil and in dust by Gaffky. The *bacillus of malignant edema* is a long, slender rod, resembling somewhat the *bacillus of anthrax*. It occurs singly, but frequently appears in long threads with irregular subdivisions; or without subdivision as long homogeneous filament. The organism is motile, possessing numerous laterally placed flagella. Motility is often absent. Oval spores are found irregularly placed in the center or slightly nearer the end and cause a bulging of the body. It stains readily by the usual aniline dyes but is decolorized by Gram.

The organism seems to be pathogenic for all animals. Subcutaneous inoculation of pure cultures produces an acute edematous inflammation at the point of inoculation within 24 to 36 hours. This edema spreads throughout the subcuticular and deeper layers and consists of a thin fluid which is slightly bloody. The lymphnodes become enlarged and hemorrhagic. Gas is formed causing subcutaneous emphysema. The toxemia is general, and in small animals the disease is usually fatal. At autopsy the organisms are found in the edema about the local lesion. The organism is not found in the blood or internal organs shortly after death, though later they may become distributed throughout the body. The internal organs show parenchymatous degeneration with occasional hemorrhages.

Infection with malignant edema is rare. It has been seen in horses, in cattle and in sheep. Infection generally takes place after trauma, and in man

secondarily, after compound fractures, or upon the site of suppurating wounds.

A recovery from the infection will produce immunity. Immunity may also be produced by the injection of soluble toxin in bacteria-free filtrates of the organism. Very small amount of this toxin is capable of killing guinea pigs. Immunity can also be produced by injection with the toxic filtered sera of animals dead from the disease. The bacillus of malignant edema may be cultivated under strict anaerobic conditions upon any of the ordinary media. The addition of glucose to the media will favor its growth. Gases are produced through proteid cleavage. On gelatin plates, small, grey, spherical colonies, with microscopic radial filaments, appear in about 3 days. The gelatin is liquefied. In the gelatin stab, growth appears along the entire stab to within a short distance of the surface. Processes develop laterally with a formation of gas. In agar, the growth appears as a white line along the entire length of the stab, which takes on a lateral cloud-like extension, and in the presence of sugar, bubbles will be formed throughout the medium. In broth, there is general clouding and a granular precipitate. Milk is slowly coagulated.

BACILLUS AEROGENES CAPSULATUS.

The *Bacillus Aerogenes Capsulatus* is found in soil, dust and brackish water and in the intestine of human beings and animals. They were first observed by Welch in 1892, having obtained it during autopsy from the blood of a case of ruptured aortic aneurysm. His attention was called to the blood by reason of air bubbles appearing throughout the vessels.

The organism appears as a straight rod of varying length, and somewhat thicker but not unlike the anthrax bacillus. On rare occasions the organism may be slightly curved, and still rarer it may be so short as to appear almost coccoid. The organism generally appears singly with rounded ends, but may appear as short chains

when the ends are almost square. The chain formation takes place in the blood. Long chains are never seen in artificial culture media which distinguishes the organism from the bacillus of anthrax. Spores are seen in blood serum cultures, rarely upon plain agar and never in the animal body. They are oval in form and may be centrally or polarly located. It is non motile and enclosed by a capsule which cannot always be demonstrated. The capsules are best seen when preparations are made from animal fluid, though they may be seen in specimens grown upon artificial culture media. It is stained readily by the ordinary aniline dyes and by Gram, if taken from tissues. If taken from artificial culture media, they are partially decolorized by Gram by reason of involution forms.

The bacillus is highly pathogenic for guinea pigs but very slightly so for rabbits. The virulence varies with the strain. In general, its pathogenicity for laboratory animals is slight. The bacillus has been isolated from numerous cases of emphysematous gangrene in man, which is characterized by a rapidly necrotizing inflammation accompanied by subcutaneous emphysema. Infection, when it takes place, follows trauma, especially compound fractures. The organism has also been found in the uterus in puerperal infection. It has also been found in infectious processes of gastrointestinal and biliary tracts, the lungs, the pleura and the meninges.

The organism may be isolated from mixed cultures by animal inoculation. About 1 cc. of the suspected material is emulsified with about 5 cc. of sterile salt solution and filtered through sterile paper. 1 to 2 cc. of this suspension is injected into the ear vein of a rabbit. The rabbit is then killed and placed in the incubator for five to eight hours, at the end of which times the carcass will be found to be distended with gas. Autopsy will show gas to be distributed as bubbles throughout the organs. From these bubbles, the organism may be taken for identification or culture. The organism is grown under obligatory an-

aerobic conditions upon any of the usual media of a neutral or slightly alkaline reaction. The addition of glucose or lactose will favor the growth. Upon plate cultures a flat, grayish, translucent, round colony with slightly irregular and fringed margin appears within 24 hours. Gelatin is slowly liquefied, but occasionally liquefaction does not occur. In sugared agar stabs or slants, there is a rapid formation of gas which is considered of diagnostic value. In broth, there is general clouding, and within 40 hours a heavy, white flocculent sediment is formed. Froth appears upon the surface of the broth tubes, if undisturbed, due to the formation of gas. On potato, the growth is scanty. On coagulated blood serum, the growth is heavy with slight peptonization of the medium. Milk is rapidly coagulated and rapidly acidified. Gas is formed.

THE BACILLUS ENTERITIDIS SPOROGENES.

This organism closely resembles the bacillus aerogenes capsulatus. It is a spore bearing organism usually present in the intestinal tract of man. It is found in sewage, milk, dust and food stuffs, such as wheat, oatmeal, rice, etc. Cline believed that it produced diarrhea when taken in milk. This fact is disputed by many.

BACILLUS BOTULINUS.

The Bacillus Botulinus was discovered by van Ermengen, in 1896, who isolated the organism from a ham, the eating of which had caused disease in a number of persons. He found the bacilli within the muscle fibers of the ham in great numbers and was also able to cultivate the same microorganism from the stomach and spleen of one of the individuals who died from the infection. The bacillus is a large, straight rod with rounded ends, appearing singly or grouped in very short chains. It is slightly motile and develops oval spores which are situated near the end of the bacillus, on rare occasions at the middle. It is stained readily by the ordinary aniline dyes also by

Gram, if care be taken that there is not over decolorization with the alcohol.

Ingestion of meat infected with this organism produces the botulism or allantiasis in man. After a period of incubation of from 24 to 48 hours, the symptoms are: chilliness, trembling, giddiness, followed by headache, occasionally by vomiting. The chief diagnostic symptoms are due to the toxic interference with the cranial nerves, producing loss of accommodation, dilated pupils, ptosis, aphonia and dysphagia, etc. Fever is usually absent. Consciousness is rarely lost. These symptoms differ from the meat poisonings caused by other micro-organisms.

Animals inoculated with living cultures or toxins will show the symptoms indicated above. Guinea pigs seem to be the most susceptible. They are killed by injections of minute quantities of the toxin. Before death, which occurs within 24 to 36 hours, general motor paralysis, dyspnea, and hypersecretion of mucus from nose and mouth may occur.

Autopsies show a general hyperemia of the organs with much parenchymatous degeneration and many minute hemorrhages.

The poisoning occurring in man is due to the toxins that have been formed by the bacillus in the ingested meat. It has been shown that little or no toxin is produced by the bacillus after it has been introduced into the animal body. It produces the disease by the absorption of the toxin that is secreted by the organisms. This toxin is active not only when injected subcutaneously, but also when introduced through the gastrointestinal canal. A specific antitoxin has been produced by Kempner.

The organism is easily cultivated under strict anaerobic conditions in the ordinary culture media of a neutral or moderately alkaline reaction and at a temperature not exceeding 35° C. On gelatin, the growth is rapid and abundant with the formation of gas and rapid liquefaction. On glucose gelatin plates, the colonies appear as round, yellowish, transparent spots

surrounded by a zone of liquefaction. On agar plates, the colonies are yellowish, opalescent and round in shape, with a finely fringed border. Stab cultures in glucose agar produce a thin white growth along the line of stab which does not reach the surface of the medium. The medium is split by the formation of gas. Milk is not coagulated. Disaccharides and polysaccharides are not fermented. The gas that is formed in cultures, chiefly hydrogen and methane.

HEMORRHAGIC SEPTICEMIA GROUP OF ORGANISMS.

BACTERIUM PESTIS.

(Bacillus of Bubonic Plague).

The bacterium of bubonic plague was described by Yerdin and Kitasato in 1893, independently of each other, and is now recognized as the etiological factor in bubonic plague, epidemics of which have been recognized since the second century. About half the population of the Roman Empire died in the sixth century, and during the fourteenth century an epidemic (The Black Death) swept over Europe and killed about twenty-five million people. About two million died of this disease in India during 1901 to 1904. Smaller epidemics have appeared in numerous parts of the world, as China, Egypt and South Africa.

The organism is a short, thick bacillus with well-rounded ends. It is non-motile, and appears singly, in pairs and occasionally in short chains. The organism shows distinct polar stain. The size of the organism varies, and in old cultures involution forms may appear either as club-shaped diphtheria-like bacilli or as swollen coccoid forms. The involution forms are of diagnostic importance by reason of their irregularity, and seem to be more numerous in artificial media. There are no spores present, and certain observers have demonstrated a gelatinous capsule. Occasionally, branched forms may be observed. It stains easily with aniline dyes, particularly, at the poles. It is Gram negative.

The organism is extremely pathogenic for rats, mice, guinea pigs, rabbits and monkeys. Even insects die from the infection. Among animals, the disease has been found chiefly in rats and squirrels. Dogs may occasionally become infected. Two distinct types are observed clinically, depending upon the mode of infection, which takes place by the entrance of organism through the skin or by the respiratory tract. In the cutaneous infection, which may take place through the most minute lesion, the disease is first localized in the nearest lymphnodes, and from these primary dwellings the bacilli may enter the blood and produce secondary foci. If entrance has taken place through the respiratory tract, a pneumonia is produced which usually proves fatal within four or five days. Cardiac depression is a very characteristic symptom of systemic infection. The diagnosis may be made during life by finding the bacilli in the aspirated fluid from a bubo, or from the sputum. Identification is made morphologically, culturally, animal inoculation and agglutination tests.

At autopsy, in man, the bacilli are found in the primary lesion in the blood and in the spleen, the liver and lymphatics. Hemorrhages into the serous cavities may be found. The pneumonic type is usually of the bronchopneumonic variety, with extensive swelling of the bronchial lymphnodes. When the disease has been prolonged, tubercle-like foci may be found in the spleen, liver and lung.

The typical lesions found in rats, who play an important role in the spread of the disease, becoming infected from the cadavers of plague victims or the eating of the bodies of rats, dead from the disease, are engorgement of the subcutaneous vessels and a pink colorization of the muscles. The bubo when present is sufficient for diagnosis. The area surrounding the buboes are markedly injected and sometimes hemorrhagic. A pleural infusion is present. The liver has undergone a fatty change. The spleen is large, friable and often presents pinpoint granules on the surface.

The systemic symptoms are due to the absorption of the toxin, which are of the endotoxin and also of a true soluble toxin variety.

A single attack will immunize. The antibodies developed are agglutinins, bacteriolytic and possibly antitoxin. The agglutinins are of importance in diagnosis. Active immunization is accomplished by the inoculation of the whole dead bacteria. The serum of immunized animals has been tried as a therapeutic agent and gives encouraging results when administered early.

The bacillus is isolated in pure culture from the lesion during life or at autopsy. The organism grows readily, best upon meat infusion of a neutral or moderate alkaline reaction and at a temperature of 30° C. It will grow at temperatures ranging from 20° to 38° C. On agar, minute colonies with a compact center surrounded by an irregular, indented, granular margin appears within 24 hours. On gelatin, colonies like those upon agar appear after two or three days. It is not liquefied. In bouillon, the organism grows slowly, sinking to the bottom or adhering to the sides of the tube as granular deposit. Occasionally a delicate pellicle is formed. Milk is not coagulated. In litmus milk, there is slight acid formation. No indol is formed upon peptone.

The organisms are eliminated in the exudates from surrounding buboes and from the sputum in the pneumonic type and are present throughout the body after death; therefore, the dead bodies of human beings and of rats are sources of infection for other rats, which become chronic carriers of the disease; and even though showing no symptoms themselves, they must necessarily be important factors in the maintenance and spread of the disease.

If in a dark and moist environment, the organism may live outside of the body for months and even years. In cadavers, they may live for weeks and months, if protected from dryness. Complete drying will kill the organism in two or three days, and

dried artificially, they may be killed within four or five hours. A dry heat of 100° C will kill the organism in one hour, boiling the organism will kill it in a few minutes. They are very resistant to cold. Direct sunlight destroys them within four or five hours. They are not very resistant to antiseptics.

Prophylaxis consists in isolating infected animals, followed by thorough disinfection, involving even the killing of fleas and the destruction of rats, squirrels or other animals which may serve as carriers. Haffkine's vaccination method has also been shown to be a valuable prophylactic measure.

BACTERIUM TULARENSE.

McCoy has described a disease which occurs in the California ground squirrel which closely resembles, so far as the lesions are concerned, the plague infection of these animals. McCoy was able to transmit plague-like lesions in most animals inoculated with infected material, and later, in 1912, McCoy and Chapman isolated the bacterium on an egg medium. The organism is small and often capsulated, staining poorly with methylene blue, better with carbo fuchsin or gentian violet.

BACTERIUM AVISEPTICUS.

(Bacillus of Chicken Cholera).

The bacillus of chicken cholera was discovered in the blood of infected animals by Pasteur in 1880. This disease is widely prevalent in chickens, ducks, geese and a large variety of smaller birds. The organism is a short non-motile rod, staining easily with analine dyes. It is decolorized by Gram. It often appears as a diplococcus by reason of marked polar staining qualities. Spores are not formed. Occasionally vacuolated forms, not unlike those of the bacillus pestis, occur. The infection of birds is extremely acute, accompanied by diarrhea, often bloody stools, great exhaustion, drowsiness, and ending fatally within a few days. Autopsy shows hemorrhagic infiltration of the

intestinal mucosa, enlargement of the liver and spleen and frequently a bronchopneumonia.

The bacilli may be found in the blood, in the organs and in the exudates, if present, and in large numbers in the dejecta. It grows well upon the ordinary culture media at a temperature of 25° to 40° C. Broth is clouded uniformly with a later formation of a pellicle. Upon agar, minute white or yellowish, first transparent, later, opaque colonies appear within 24 to 48 hours. No liquefaction of gelatin. No coagulation of milk. No gas is formed in sugar broth, which, however, becomes acid, and in peptone, indol is formed.

Infection takes place probably through food and water contaminated by discharges of diseased birds. The feeding or subcutaneous inoculation with cultures, even in the most minute dose, will produce a quickly developing septicemia, which is uniformly fatal.

The bacillus is extremely pathogenic for rabbits, if subcutaneous inoculations are made; less so for hogs, sheep and horses. The disease does not follow the ingestion of infected material by these animals.

BACTERIUM SUISEPTICUS.

(Bacillus of Swine Plague).

The bacillus of swine plague causes an epidemic disease among hogs characterized by a bronchopneumonia, which is followed by a general septicemia. A pleural exudate of a sero-sanguineous nature, together with enlargements of the bronchial lymph glands, the liver and the spleen, is frequently found. The gastrointestinal tract is rarely involved. At autopsy a nonmotile Gram negative bacillus, almost identical with the bacillus of chicken cholera, may be found in the lungs, in the exudates, in the liver, spleen and in the blood. The disease is seldom acute, but is almost uniformly fatal in young pigs. Spontaneous infection usually occurs by inhalation. Experimentally the disease has been produced by subcutaneous inoculation. Mice, guinea

pigs and rabbits inoculated subcutaneously with small doses of the organism are killed within four or five days. Active and passive immunization has been successful.

Kitt and Mayr have shown that the serum of horses immunized with chicken cholera would sometimes protect against bacillus suissepticus.

BACTERIUM BOVISEPTIUM.

The bacterium bovisseptium produces a disease and affects a wide variety of domestic and wild animals. It has been reported from many portions of North America, some sections of South America and many European countries, and is known as Corn Stalk disease, Buffalo disease and pneumonenteritis, etc. The domestic animals most commonly affected are cattle, sheep, horses and goats. The onset of the disease is sudden and the case acute. It does not spread from herd to herd, but appears in isolated outbreaks. Body infection probably occurs by inoculation. The characteristic lesions found at autopsy are hemorrhages, which occur subcutaneously under the mucus membrane or under the serous membrane and also in the lymph glands. The lesions produced by this bacterium indicate a general distribution through the body.

The mortality ranges from 50 to 80 per cent. The acute and rapidly fatal cases, where the autopsy shows only trifling lesions, would indicate the formation of active toxins.

Very little is known concerning the elimination of this organism from the diseased body, but isolation and disinfection are to be recommended on general principles.

The disease resembles anthrax and symptomatic anthrax in some of its characteristics. It may be readily differentiated from either of these diseases by microscopic examination.

The organism resembles the bacterium of chicken cholera, of rabbit septicemia and the bacillus of cholera suissepticus so closely that laboratory differentiations are extremely difficult. The bacterium is small, with rounded ends, closely resembling a diplococ-

cus. Involution forms may appear. Shows by polar staining, decolorizes by Gram, produces no spores, has no flagella and is nonmotile.

THE COLON-TYPHOID DYSENTERY GROUP OF BACILLI.

The organisms belonging to this group present great differences in their pathogenic characters, but possess so many points of similarity in their morphological and biological characteristics that their differentiation becomes extremely difficult. The group considered will be the Coli group, the Enteritidis group, the Dysentery group and the Typhosus group.

The differentiation of these organisms is important in that certain of the bacilli are specifically pathogenic, while others are essentially saprophytic and become pathogenic only under exceptional conditions.

BACILLUS COLI GROUP.

These organisms, sometimes called "Lactose Fermenters," are frequently nonpathogenic for man but may become distinctly pathogenic under certain conditions. Their degree of virulence upon inoculation of lower animals varies greatly.

Motility is not marked, or none. Dextrose and lactose are fermented with acid production. Milk is quickly coagulated with acid production. Indol is not produced by most varieties.

THE BACILLUS COLI.

Under the name of Colon Bacilli are grouped a number of varieties which differ from one another in minor characteristics, but correspond in certain cardinal points, which warrants their consideration under one heading.

The **Bacillus Coli Communis** is the most prominent type of the group, and therefore will be chiefly considered. This organism was discovered by Buchner in 1885. It is a constant inhabitant of the intestinal canal of human beings and animals. It is occasionally found in the soil, air, water and in milk. It is, in fact, found in all thickly populated neigh-

borhoods. In man, the bacillus coli appears normally in the intestine, being found in greatest numbers at or about the ileocecal valve diminishing from here upward to the duodenum and downward as far as the rectum. The organism is frequently found in the tissues and in blood after death without visible lesions of the intestinal mucosa. It is probable that it may enter the circulation a few hours before death. Extensive investigations have been carried out to determine whether or not the presence of the organism in the intestines possesses a definite physiological function of advantage to its host. This question has not been definitely settled though it might be stated that the function of the organism in the intestine is not inconsiderable if only because of its possible antagonism to certain putrefactive bacteria, as has been demonstrated by Bienstock. The bacillus coli communis is a short rod, varying in thickness from one-third to one-fifth its length. Under certain conditions of cultivation, it may appear more slender or shorter and even coccoid in form. It usually appears singly, but occasionally in short chains. There are no spores. When first isolated from the body, it may be extremely motile, while old laboratory strains of the organism may show almost no motility. Ordinarily the motility of the colon bacillus is intermediate between these two extremes. Stains readily with the ordinary aniline dyes and is decolorized by Gram.

The pathogenicity of the organism for animals is slight and varies with the different strains. If 1 cc. of a bouillon culture is injected intraperitoneally into guinea pigs, death will often ensue. If large doses are injected intravenously into rabbits, symptoms of violent intoxication are presented, followed by death in from 24 to 48 hours. Moderate doses inoculated subcutaneously usually produce nothing more than a local abscess, from which the animal recovers. It is probable that death results from the action of poisons liberated from the disintegrating bac-

teria, and not from multiplication of the bacilli themselves.

In man a large variety of lesions produced by the organisms have been described. The manner in which the organism becomes pathogenic is not clear. A number of explanations have been advanced. First, that whenever an infection is produced by the bacillus coli, it is produced by one that has been recently acquired from another host; second, that the virulence of the organism may be enhanced by inflammatory processes brought about by other organisms; third, infection may possibly take place by reason of a reduction in the resistance of the host. Whatever the cause for the infection, it is doubtful whether septicemia produced by the colon bacillus is due to an actual primary invasion of circulation by the bacillus, although a few unquestionable cases have been reported. Diseases, such as cholera nostras and cholera infantum, have been attributed to these organisms, but without being supported with satisfactory evidence. It is likely that in most of the intestinal diseases formerly attributed to these organisms the organism plays but a secondary part. In peritonitis following perforation in the intestine, the organism is always present, but can never be found in pure culture, being usually accompanied by staphylococci and streptococci and other micro-organisms. It is therefore hard to determine whether or not these bacilli could be considered as a primary cause of peritonitis. The organism may give rise to a mild suppurative process, in as much as it is able to proliferate within the peritoneum. Welch reports a case of peritonitis in which the bacillus coli was isolated in pure culture. The organism has also been isolated from liver abscesses from the bile and from the center of gall stones, consequently inflammatory conditions have been attributed to it in these situations.

The organism is found more frequently in the urine than any other organism. It may be present in normal individuals. It is frequently seen during

convalescence from typhoid. It may disappear spontaneously or cystitis may supervene and occasionally an ascending pyonephrosis. The organism may cause localized suppurations in all parts of the body, most frequently seen, however, about the anus and the genitals.

The toxic action of the colon bacillus is due to endotoxins. The injection of gradually increased doses of living or dead colon bacilli will produce specific bacteriolytic agglutinating and precipitating substances. The injection of any specific race of colon bacilli produces in the immunized animal high agglutination value only for the individual culture used for immunization.

The large number of varieties of colon bacilli described during the early days of bacteriology were, in many cases, based upon a temporary depression of one or another cultural characteristic, although some were undoubtedly closely related. They were, however, distinct groups.

The constant and distinct varieties of the bacillus coli do not occur. The most common is the **bacillus coli communior** (Dunham), and is believed to be more abundant in the human and animal intestines than the coli communis itself. It possesses all the cardinal characteristics of the colon group. It differs, however, from the bacillus coli communis, in that it produces acid from the saccharose as well as from dextrose and lactose; whereas the coli communis does not form acid or gas from saccharose.

The Bacillus Coli Communis is an aerobe and facultative anaerobe. It will grow upon all of the ordinary media at a temperature ranging from 20° to 40° C, optimum 37½° C. In broth, general clouding with later pellicle and a light, slimy sediment is formed. Upon agar, gray colonies appear within 12 to 18 hours, which gradually become more and more opaque. The surface colonies often show a characteristic grape leaf structure, or may be round and flat and show a definitely raised, glistening surface. Upon agar slant, the growth occurs in a uniform layer. On

gelatin, the growth is rapid with no liquefaction. On potato, the growth is abundant and of a gray white, glistening layer which later turns to a yellowish brown, and in old cultures often to a dirty greenish brown. Indol is formed in peptone solution. Milk is acidified and coagulated. In lactose litmus agar, acid is formed and the medium becomes red. Gas bubbles appear along the line of the stab inoculation. Gas is formed in dextrose, lactose and mannite, but not in saccharose. Acid and gas are formed in levulose, lactose and maltose.

The isolation of the colon bacillus from mixed cultures is accomplished by plating upon lactose litmus agar. Cultures of the colon bacillus are characterized by an odor not unlike that of diluted feces. The acids formed by the organism from sugar are lactic, acetic and formic acids. The gas produced is chiefly carbon dioxide and hydrogen.

BACTERIUM LACTUS AEROGENES.

(*Bacillus Aerogenes*).

This organism was first isolated by Escherich (1885) from the feces of infants. It is almost constantly present in milk, and together with one or two other microorganisms, is the chief cause of the ordinary souring of milk. It is also widely distributed in nature in feces, in water and in sewers. It is an anaerobe and facultative anaerobe. Distinguished from the colon bacillus in that it is non-motile, very seldom forms chains, and when cultivated in milk it possesses a distinct capsule; also in that it will ferment polysaccharides as starch and does not produce indol.

The organism is but slightly pathogenic to man. It is almost constantly in the human intestines. In infants it may give rise to flatulence and has been known to produce cystitis. In rare instances it has formed gas in the blood. The different strains of the organism vary in their pathogenicity for animals.

It grows abundantly at a temperature between 25° to 30° C on all the ordi-

nary culture media. Upon agar and gelatin, heavy, white, mucoid colonies appear which have a tendency to confluence. In broth, there is general clouding and pellicle formations; a sour or cheesy odor. Upon potato, growth is heavy and gas is formed. Milk, coagulated and acidified. The organism's chief characteristic is that it is capable of producing a large amount of acid, chiefly lactic, and of being able to withstand this quantity of acid without being injured. All carbohydrates, except saccharose, are fermented with the formation of gas.

BACILLUS MUCOSUS CAPSULATUS.

(Friedlander's Bacillus. Bacterium Pneumonia, Pneumobacillus).

Friedlander's Bacillus, the Bacillus of Rhinoscleroma, the Bacillus of Ozena, etc., together with a number of bacteria reported as allied to Friedlander's, mainly upon morphological grounds, are classified together as the "Friedlander group" of organisms.

This organism was discovered by Friedlander in 1882, and believed by him to be the cause of lobar pneumonia. He described it as a micrococcus. Researches by Frankel and Weichselbaum proved this to be a short, incapsulated bacillus, which occurred in lobar pneumonia on rare occasions only. The organism is a short, plump bacillus with rounded ends, varying greatly in size, even in the same culture. In animal and human lesions the organism is almost coccoid in form. It appears singly in diploform or in short chains. They are nonmotile. It is surrounded by a capsule in animals when taken from animal fluid and sometimes in the smears from agar or gelatin. The capsule is two or three times the size of the bacillus, and when seen in chains or in groups, several bacilli may be enclosed within one capsule. The capsule will disappear in prolonged cultivation on agar or gelatin. It stains easily with the ordinary stains, but is decolorized by Gram.

It causes pneumonia probably in about 7 or 8 per cent. of all cases in this

country. When causing the disease, the pneumonia is extremely severe and usually fatal. It has been found in ulcerative stomatitis and nasal catarrh. It has been reported as occurring in severe tonsillitis in children, in the pus from suppurations in the antrum of Highmore and nasal sinuses and in cases of ozena, believed by Abel to be the specific cause. It has been found in empyemic fluid, in pericardial exudate and in spinal fluid. Cases of septicemia have been described, caused by Friedlander's bacillus. It has been believed to be associated with some forms of diarrheal enteritis in that it is an occasional inhabitant of the normal intestine.

It is pathogenic for mice and guinea pigs, also for rabbits. Inoculated into susceptible animals, it will produce inflammation and death by septicemia. Intraperitoneal inoculation, a mucoid stringy exudate is found which is characteristic.

Immunization with graded doses of dead bacillus has been produced in isolated cases. Specific agglutinins in the immune serum have occasionally been found, potent only against the particular strains used. The organism is an aerobe and facultative anaerobe and grows easily on all culture media. Temperature ranges from 18° to 40° C, optimum 37½° C. On agar, grayish white mucous light colonies of a slimy, semifluid consistency appear. After three or four days a tendency to confluence causes a large part of the surface to be covered with a film of glistening, sticky exudate, which, if fished, comes off in a tenacious, stringy matter. In broth, there is rapid abundant growth with pellicle formation. General clouding and later a stringy sediment. Stab cultures in gelatin show at first a white line of growth along the course of the puncture; later, there appears a grayish glucose droplet on the surface, which enlarges and gives the growth a nail-like appearance, which is regarded as diagnostic. The gelatin is not liquefied. On potato, an abundant growth appears of a slightly brown color. There is no

indol formed in peptone solution. In milk, the growth is rapid, with irregular coagulation. All carbohydrates (except lactose) are fermented with formation of gas.

BACILLUS OF RHINOSCLEROMA.

The bacillus was discovered by von Frisch in 1882. It produces a disease called in rhinoscleroma in man, which consists of a slowly grown granulomatous inflammation, located at the external larynx, or the mucosa of nose, mouth, pharynx or larynx. Within the interior of the lesions are many large, swollen cells, within which the bacilli lie; also in the intracellular spaces. The disease is rare in America. It is slowly progressive and comparatively intractable to surgical treatment, seldom affecting the general health unless by obstruction to the air passages. The bacillus may be isolated from the lesions. Morphologically and culturally, it is almost identical with Friedlander's bacillus. It differs from Friedlander's bacillus in forming no gas in dextrose bouillon, and producing no acid in lactose bouillon and never coagulating milk.

BACILLUS OZENA.

Abel and others have shown that an organism morphologically and culturally almost identical with the bacillus mucosus capsulatus is nearly always present in ozena. While he states that it does not form gas in dextrose bouillon and is less pathogenic for mice than is the bacillus of Friedlander, it cannot be definitely stated as to whether it is a separate species or merely an atypical form of the bacillus of Friedlander.

BACILLUS ENTERITIDIS GROUP.

Most members of this group of organisms are under certain conditions distinctly pathogenic for many of the lower animals and for man. The motility is usually marked. Dextrose is fermented with gas formation. Lactose is not fermented. Milk is not coagulated. No indol or only a slight amount is produced (bacillus alkaligenese does not ferment sugars).

Gartner's discovery in 1888 of the bacillus enteritidis in association with epidemics of meat poisoning gave impetus to the study of a number of bacteria resembling in many characteristics the colon or typhoid bacilli.

They are often spoken of as "group of intermediates" and classified as intermediate between the colon and the typhoid types.

By reason of the pathological conditions with which they have been associated, the terms "hog-cholera group," "enteritidis group," "paracolon group" or "paratyphoid group," were applied to the chief members under investigation.

The microorganisms are morphologically indistinguishable from the colon and typhoid bacilli. Pathogenically they have attracted attention in their connection with meat poisoning and with protracted fevers that are indistinguishable from mild typhoidal infections.

Bacillus Enteritidis (Gartner) was discovered and isolated from the meat of a cow, the ingestion of which had produced the symptoms of acute gastrointestinal catarrh in fifty-seven people. The organism was demonstrated in the spleen and in the blood of one patient who died from the disease. The organism is actively motile, forms no indol and produces gas in dextrose media. If fed to mice, guinea pigs, rabbits and sheep, it will induce acute gastrointestinal symptoms. The bodies of the organisms contain an extremely toxic substance which differs from the endotoxins of other bacteria in that it is extremely resistant to heat. Sterilized cultures have the same pathogenic effect as the living cultures.

Bacillus Morselle, described by Van Ermengem in 1891 in an epidemic of meat poisoning at Morselle, differing slightly in minor characteristics, is almost identical with Gartner's bacillus.

Bacillus Psittacosis, isolated by Nocard in 1892 from infections in parrots, showed a close resemblance to Gartner's bacillus.

Bacillus Typhimurium, isolated by Loeffler, was in 1893 shown to be similar to Gartner's bacillus and also to the so-called "hog-cholera bacillus," by T. Smith and Moore from their studies of the disease of swine. They first used the term of "hog cholera" group.

Paracolon and Paratyphoid Group were introduced by Gilbert in 1893 to designate the organisms of this group resembling more nearly the biological characters, the colon bacillus on the one hand and typhoid bacillus on the other.

Bacillus bovis morbificans was isolated by Basenan in 1894 in an epidemic of meat poisoning; differing slightly in minor characteristics, it is almost identical with Gartner's bacillus.

Paracolon Bacillus was isolated by Widal and Nobecourt in 1897 from an esophageal abscess following typhoid fever. This organism showed a close resemblance to Gartner's bacillus, and following the Gilberts' suggestion they named it "B. paracolon."

Gwyn, in 1898, isolated an organism from the blood of a patient who presented all the symptoms of typhoid fever, but the patient's serum did not have any agglutinating power for the bacillus typhosus. Its culture characteristics were similar to those of Gartner's bacillus which was agglutinated by the patient's serum. He called it "paracolon bacillus." The paracolon and paratyphoid can be distinguished without difficulty from the typhoid bacillus. They produce gas in glucose media, and in this respect they differ from typhoid, but, unlike *B. coli*, they do not produce gas from lactose, coagulated milk, or, as a rule, from indol.

Agglutination tests applied to the intermediates show that the members of the paracolon group do not all show mutual reactions, and the group, like the *B. coli*, is therefore composed of a number of distinct races. The paratyphoids, most of which have been isolated from cases simulating typhoid fever, belong chiefly to two strains. An active serum prepared from either strain of

the bacilli will agglutinate all the others of that strain. They are designated as type A and type B.

A similar organism was isolated by Cushing, in 1900, from a costochondral abscess during convalescence from typhoid fever.

Bacillus Icteroides, associated by Sana-relli with yellow fever, was shown by Reed and Varroll, in 1899, to be culturally similar to the bacillus of hog-cholera.

Bacillus Paratyphoid. Schottmuller, in 1900, isolated bacilli from five cases which corresponded to bacilli previously described.

Cultural and agglutination studies of the organisms obtained showed that they could be divided into two similar yet distinctly different types; one of them very close to the typhoid type (B. Paratyphoid); the other, closer to the Gartner bacillus.

Type A has been isolated from the normal intestines of animals by Morgan and is not considered very important as a causative agent of human disease. Kutscher therefore suggests that, except in rare instances, this organism is a nonsaprophytic parasite. Type B not infrequently produces an infection.

Clinically, the diseases caused by this class of bacteria may be divided into:

Group 1. Those which fall into the category of meat poisoning (Paracolon) more like those due to B. Gartner, having sudden, violent onset of gastrointestinal symptoms directly following the ingestion of meat, and characterized by profound toxemia.

Group 2. Those in which the disease simulates a mild form of typhoid fever, lasting from twelve to eighteen days, and differing only by the absence of the specific agglutination reaction for typhoid bacilli.

Bacillus Alkaligenes. This bacillus resembles somewhat a colon bacillus which has lost its power to ferment sugars. Morphologically and culturally it is more like the typhoid bacillus. It ferments no sugars. It is frequently present in the intestines and may have pathogenic properties.

Bacillus Cholerae Suis (Bacillus of Hog-Cholera). This organism is an ac-

tively motile bacillus; grows readily in bouillon; renders milk at first slightly acid, then strongly alkaline; dissolves casein and ferments dextrose with acid production. It is found almost regularly present in hogs, sick of cholera, but is not the essential cause of the disease. Although it is not an essential factor in exciting hog-cholera, it is believed to be of importance as an added infection. It is pathogenic for hogs, causing, when fed, a fatal enteritis. Theobald Smith and Moore, in 1893, studied this disease and noted a great similarity between the organism, the bacillus of the Gartner group and the bacillus typhi murium isolated by Loeffler.

Bacillus of Swine Plague. This is a nonmotile bacillus which grows feebly in bouillon. Does not coagulate milk and ferments glucose without production of gas. When fed to pigs it does not usually cause illness. It is closely related to the hemorrhagic septicemic group.

THE BACILLUS DYSENTERY GROUP.

This group of organisms, often grouped with bacillus typhosus are pathogenic for man and by inoculation less pathogenic for animals. The organisms are nonmotile, ferment dextrose without formation of gas, do not ferment lactose, do not coagulate milk nor produce indol. The bacillus dysenteria (Shiga) does not ferment mannite. The bacillus paradysenteria (Park) ferments maltose and mannite. The paradysenteria (Flexner) ferments mannite only.

Bacillus Dysenteria (Shiga). The etiology of dysentery was obscure until Shiga (1898) found a bacillus in the stools of patients suffering with dysentery which had not before been identified. It was present in all the cases of epidemic dysentery examined but was not found in the stools of healthy persons.

The blood of dysenteric patients agglutinated the bacilli which were isolated, but the organisms were not agglutinated, to any such degree by

serum from healthy individuals. The organism is a short rod similar to the colon group of organisms. Stains easily with the aniline dyes with the ends showing a tendency to deeper staining than the center.

It decolorizes by Gram's. No spores or flagella have been demonstrated. On gelatin the colonies appear more like the typhoid than the colon bacilli. It is not liquefied. On agar the colonies resemble those of the typhoid bacilli.

On potato, a delicate, scarcely visible, brownish growth is formed. In bouillon, a diffuse cloudiness is formed with a slight deposit appearing after some days. Occasionally a pellicle is formed.

Litmus Milk becomes a pale lilac after 24 hours, which returns to the original color after three to eight days. Neutral red agar is not changed.

It does not form indol, except, perhaps, in intestine, or ferment mannite, maltose or saccharose. Animals injected with this type of organism produce specific agglutinins which only in a small way combine with the other type of the group.

In man, the organisms produce an acute dysentery with symptoms of cramps, diarrhoea and tenesmus. The stools, at first feculent, then seromucous, become bloody or composed of coffee-ground sediment. At the height of the disease there are ten to fifty stools in the twenty-four hours. The blood usually disappears after from two to seven days. The disease is especially limited to the mucous membrane of the large intestines. The vessels of the surfaces appear congested and prominent. The mucous membrane is covered with a yellowish mucous and seems to be absent in places. The solitary follicles are enlarged, especially in the sigmoid flexure, and in some instances are depressed and appear to be necrotic in their center. Microscopically, the mucous glands are normal except over the solitary follicles, where they are slightly broken down and contain polynuclear leukocytes. The capillaries of the follicles are extremely congested. The submucosa is thick-

ened and slightly edematous. The connective tissue cells have undergone a slight hyaline degeneration. The deeper coats of the intestine are not involved.

The small intestine seems to be slightly distended. The mesenteric glands are large and red. Peyer's patches are swollen slightly, but without ulceration. Microscopically, the mucous membrane appears normal.

In the severe cases the entire lumen of the intestines may be filled with a pseudomembrane of a diphtheretic character. In young children the lesions appear to be more superficial even in fatal cases.

Animals injected intravenously with the organism show symptoms of diarrhoea and paralysis, which is followed by death. Animals are likewise very sensitive to killed cultures. Autopsies on animals killed from injections into the peritoneum of living or dead bacilli show a hyperemic peritoneum, the cavity of which is more or less filled with serous or bloody serous exudate. The liver may be covered with a fibrinous mass, the small intestine filled with fluid, the large intestine usually empty, and the mucous membrane of both hyperemic and may sometimes be hemorrhagic. Subcutaneous injections of the dead or living organisms produces infiltrations of tissue and frequently abscess formation. The organism produces both an extracellular and a cellular toxin.

Bacillus Paradysentery (Parke). "A."

In 1902, Parke and Dunham described an organism which they found in a small epidemic of dysentery occurring in men which differed somewhat from the organisms previously described, in that it produces endol. The organisms ferment mannite with the production of acid, but does not act upon maltose or saccharose. Animals injected with this organism develop immune bodies and agglutinins that are specific for this type.

Bacillus Paradysentery (Flexner). "B."

Flexner in 1899, while investigating the dysentery in the Philippines, isolated an organism which corresponds to Shiga's organism, but differs from the other in that it produces endol,

ferments mannite and acts strongly upon maltose and feebly upon saccharose. This type of organism is nearest to the Colon group.

The two mannite fermenting types are widely scattered over the world and caused epidemics of dysentery of a milder type than that produced by the Shiga organism and have been described by many investigators. These two types have also been described at times in mixed infections where dysentery symptoms are almost or entirely absent.

Passive immunization of animals and human beings with the serum of immunized horses has been attempted by Shiga, Kruse and others, who have reported a reduction of mortality by the use of such sera. Todd has demonstrated the neutralization of the solutions of toxin by an immune serum.

By reason of the different varieties of dysentery bacilli, polyvalent sera is of considerable value. It is given subcutaneously in 20 cc. doses once or twice a day for several days, or until convalescence is established.

BACILLUS TYPHOSUS GROUP.

The *Bacillus Typhosus* is an actively motile organism pathogenic for man and less pathogenic by inoculation for lower animals. Dextrose is fermented without gas formation; lactose not fermented; milk not coagulated and no formation of indol.

The organism was discovered by Ebert (1880) in the spleen and diseased areas of the intestine of typhoid cadavers.

It was obtained in pure culture by Gaffky in 1884.

The organism is a short plump rod having rounded ends. Under favorable conditions it is actively motile. The degree of motility varies in different cultures. The flagella are preipherally arranged of twelve or more in number. Many of the shorter forms have but a single terminal flagellum.

The bacillus stains a little less intensely with the ordinary aniline dyes than do most other bacteria. Bipolar

staining is sometimes marked and decolorized by Gram's.

The organism is aerobic and facultative anaerobic bacillus developing best at 37° C. Its growth is retarded above 40° C and below 30° C. Below 10° C its growth almost ceases. It does not form spores. Some of the bacilli are killed within a few hours when dried, though a few will remain alive for months under the same conditions. Usually they are killed by an exposure to 60° C for one minute.

In natural water it remains alive for 36 days (Klein).

In ice it may remain alive for three months (Prudden).

It is killed by 1-500 bichloride or 5% carbonic acid within five minutes.

Upon agar plates, small grayish colonies appear within 18 to 24 hours.

These colonies are first transparent, later they become opaque. Upon agar slants, the transparent, filiform grayish streak is formed.

Upon gelatin plates, characteristic irregular (grape-leaf) transparent, bluish-white colonies appear. Magnified, they are of homogenous structure marked by a delicate network of furrows.

As the colonies grow older they grow heavier, become more opaque and lose their early differential value.

In gelatin stab cultures the growth is mostly on the surface as a thin scalloped extension which gradually reaches the sides of the tube. In the stab proper there is but a limited growth of yellowish brown color. Gelatin is not liquefied.

Bouillon is uniformly clouded. When the medium is slightly alkaline a delicate pellicle may be formed after 18 to 24 hours' growth.

On potato, a characteristic almost invisible growth appears after 24 to 48 hours which usually covers the surface of the medium, though it may be restricted to the point of inoculation. The growth may also, however, be quite heavy and of a yellowish-brown color with a greenish halo like that of *B. Coli*. Milk is not coagulated. The neutral violet color in litmus whey becomes more red during the first 48 hours; the fluid remaining clear.

In Dunham's peptome solution there is no production of indol.

The organism does not produce gas when grown in dextrose, mannite, lactose and saccharose broth, but it does produce acid in dextrose, levulose, gelactose, mannite, maltose and dextrin broth.

In shake or stab cultures of Rothberger's neutral red no change is produced while the colon group reduce the red, decolorize the media and produce gas.

When injected into animals, no typical pathological changes are produced. The sickness or fatal results after such injections can be attributed to the toxemia produced by the endotoxins liberated from the dead bacteria.

Animals inoculated subcutaneously by bacilli, freshly obtained from typhoid cases, rapidly die.

In the peritoneal cavity they may increase with the production of a fatal peritonitis. If the bacilli are accustomed to the animal body the virulence may be so increased as to prove fatal to the animal when injected with very small cultures.

The organism produces in man an infectious disease in which the organisms pass into the blood, and by this channel they pass to all parts of the body and become localized in the tissue such as the bone marrow, lymphatic tissues and spleen, liver and kidneys.

The lesions of the intestine consist of an inflammatory enlargement of the solitary and agminated lymph nodules. In the more severe cases the hyperplasia is frequently followed by ulceration and necrosis.

Ulceration and sloughing may involve the muscular and peritoneal coats with the production of perforation. Peritonitis and death usually follows, though in rare instances adhesions may close the perforation.

The mesenteric lymph nodes undergo changes like those of the ilium. The spleen is enlarged by reason of congestion and hyperplasia. The liver and to less extent the kidney are apt to show foci of cell proliferation.

The typhoid bacillus may in rare cases act as "pus producer."

The complications occurring in typhoid fever are usually due to secondary or mixed infections with the staphylococcus, pneumococcus, streptococcus, pyocyanens and colon bacillus.

The organism is present in the urine of typhoids in about 20% of cases during the third or fourth week. When pneumonia is caused by *B. typhosus* it can be found in the sputum.

During typhoid fever the organism is always found in the gall bladder. The organism usually disappears from the body in the fourth or fifth week, but may remain for months or years in the urine and throughout life in the gall bladder.

Abscesses have been found one year after recovery from typhoid fever.

One to five per cent. of individuals having had typhoid continue to pass typhoid bacilli for years, maybe for life.

A number of so-called typhoid carriers have been reported which if not detected are very dangerous as constant spreaders of typhoid fever. The treatment of these cases has not been satisfactory. Medicinal treatment and immunization have yielded slight results.

Recovery from typhoid fever produces an immunity which may last for years, except in about 2% of cases. The second attack in these, however, is usually of a mild type.

Serum of animals immunized possesses bactericidal and feeble antitoxic properties against *B. typhosus*, and an attempt has been made to treat typhoid by this method, and although good results have been reported by a number of men the majority have found little or no value in its use.

Where danger of typhoid infection exists, the use of protective vaccines advocated by Wright are indicated. (See typhoid vaccine.)

Vaccination during the course of the fever has been advised by certain individuals, but the results obtained by Richardson does not show any effect except that relapses seem to be less.

For the diagnosis of typhoid see the Gruber-Widal reaction (Widal test).

DISEASES DUE TO THE HIGHER BACTERIA.

Leptothrix. Forms which appear as simple threads without branching. Members of this group have been found associated with certain inflammation of the mouth and pharynx. The organisms were identified by morphology.

None of the inflammations were accompanied by severe systemic symptoms and the organisms may be regarded as comparatively harmless saprophytes appearing in connection with some other specific inflammation.

Cladothrix. Thread-like forms with false branching, due to the fragmentation of the threads.

By reason of the difficulty in differentiation of this form from the streptothrix, it is likely that most cases of infection ascribed to these organisms have really been due to streptothrix infection. A rigid differentiation of true and false branching only can determine whether or not cladothrix infection may occur.

Streptothrix. (*Nocardia*.) Forms with numerous true branches and spores which usually appear in chains.

Numerous cases of infection of various parts of the body of man and animal have been reported. A member of this group has been described by Nocard as the etiological factor in a disease "farcies du boeuf," occurring among cattle in Guadeloupe.

Trevisan proposed the name "*Nocardia*" for this organism, and Wright calls attention to the misuses of the term "streptothrix" and points out that the term **Nocardia** should be used in its place.

Members of this group have also been reported in the pus from a cerebral abscess, in pulmonary disease simulating tuberculosis, suppuration of bone and of the skin and the intestinal canal. Streptothrices vary considerably in morphology. In infectious lesions they most often appear as rods and filaments with branches. Sometimes the filaments may be long and intertwined, and branch may

show club-shaped ends. Young cultures may consist of rod-shaped forms not unlike bacilli of the diphtheria group.

They stain easily with Loeffler's methylene blue or aqueous fuchsin. Cultivation upon agar and gelatin plates has been made. Grayish-white, glistening, flat colonies appear at the end of four or five days. In bouillon, a flocculent precipitate and surface pellicle is formed of the thread, without clouding.

The organisms will grow readily upon fresh, sterile kidney-tissue of rabbits. When cultures are inoculated into rabbits and guinea pigs, subcutaneous abscesses, bronchopneumonia, and suppuration, according to the mode of infection may be produced.

Actinomyces (Ray fungus) is characterized by the formation of club-shaped ends and the stellate arrangement of its threads.

The organism was first observed by Bollinger (1877) in diseased cattle and by Israel (1878) in man.

The organisms appear in the pus from discharging lesions as small granular bodies, resembling sulphur granules, visible microscopically.

They are ordinarily soft and can be easily crushed under the cover slip, but occasionally, in old lesions, they may be hard, owing to calcification.

They may be recognized easily by crushing the granules under the cover glass, and examining them, unstained, with the microscope. Fresh specimens may also be stained by Gram's.

The colony as it appears in tissue sections or pus smear consists of a rosette arrangement. The central portion of the colony is a dense mass of mycelium and spherical bodies. From this felted central mass there extends ray or club-like bodies. Club-shaped enlargements at the end of filaments frequently appear and are regarded as a distinguishing characteristic of actinomyces.

The organism grows on a variety of media. On glycerine agar the colonies develop into transparent drop-like bodies in four or five days at 37°. Old colonies become white or yellowish with a powdery surface. Some va-

rieties appear distinctly aerobic and others anaerobic. Gelatin is nearly always liquefied.

In artificial culture filaments appear which are very long and slender. They show true branching, but have no septa.

The young colony is a loose mass of filaments; older colonies become dense and fertile. Rod-shaped and spherical forms may also appear and some filaments develop conidia.

Tissue sections, stained with carmine, followed by Gram's or Wigert's, give good results.

Actinomycosis (lumpy jaw, wooden tongue) is an infectious disease which spreads rapidly. Cattle are most commonly affected, but humans, horses, sheep and dogs are susceptible.

The disease usually runs a chronic course and is distinguished especially by enlargement of affected parts, by hardening of the tongue and by suppuration.

Head parts, including the facial bones, are commonly affected; lungs and various other internal organs and even the vertebrae may be involved. The extent of injury depends upon the location and the size of the involved area.

There are several varieties of actinomyces, and it is probably specific in its relation to the disease, but it is frequently aided by pus-producing bacteria.

It is, vegetative on various grasses especially wild barley, and infection occurs by inoculation with the awns and barbs of such grasses through the mucous membrane of the mouth or alimentary tract. Infection by inhalation may occur.

It is probable that some special stage of development is necessary either within the diseased body or upon some plants in order that it may infect animals' bodies, as direct inoculation with pus usually gives negative results. Inoculation with pieces of diseased tissue occasionally gives positive results.

The pus scattered over fodder, mangers and feed racks probably serves indirectly as a source of dissemination.

An active toxin is evidently not produced. The disturbance caused by the disease is apparently due to harmful growths in the tissue and to secondary infection.

Suppuration is one of the conspicuous features, as is also the development of much new granulation tissue which tends to degenerate at the center. Soft organs affected show a tendency to multiple abscesses.

Actinobacillosis is probably to be distinguished from actinomycosis. It is very similar in history and clinical evidence but apparently different as to specific cause. The cause of actinobacillosis seems to be a bacterium found also in rosette-like masses resembling those of actinomycosis.

MYCETOMA.

(Madura Foot).

This disease is very much like actinomycosis. It is more or less limited to warmer climates; India, especially Madura. It consists of a chronic productive inflammation, most frequently attacking the foot, less often the hand, very rarely other parts of the body.

Nodular swellings occur, which break down and lead to abscess formation and later to sinuses which discharge purulent fluid containing the characteristic hard, brittle, black, granular bodies resembling grains of gunpowder. The granules may be grayish white or yellow and soft and grumous.

The appearance of these granules gives rise to two varieties of the disease:

The melanoid variety is caused by a member of the hyphomycetes group.

The ochroid variety is believed by many to be actinomycosis.

The bones are often involved and a rarefying osteitis results.

In broth, the growth is rapid, composed of long hyphae, which form a structure of a powder puff appearance.

On agar, at the end of a week, a thick grayish meshwork of hyphae spread over the surface. In old cultures, black granules appear among the mycelial meshes.

On potato a dense, velvety membrane appears with a pale brown center and a white periphery. Brown drops appear in old cultures.

PATHOGENIC MOULDS.

(Hyphomycetes, Eumycetes).

The relation of moulds to bacteria shows them to occupy a place higher than the higher bacteria which they resemble, in that they grow in filaments, but show in majority of cases a more complicated structure in possessing a more distinct wall and a definite nucleus and in their reproductive organs. The hyphae branch and grow into a network called mycelium.

In the lower forms each hypha is a single cell, septa only occurring when fructification begins.

In the higher forms the filaments are made up of rows of cells.

Most forms produce endospores in a spore sac (sporangia) situated at the end of a hypha.

Certain varieties have a primitive sexual process, a conjugation of two cells with the formation of a zygo-spore, from which a sporangium carrier may arise and develop a sporangium.

Spores may also be produced in so-called gummae (chlamydospores), which are swollen portions, segmented in the course of a hypha.

Spores may be formed as conidia. The common molds grow easily on artificial media and therefore are very apt to infect the media during bacteriological cultivations. They grow especially well in acid medium and are therefore very often found on fruit.

The majority of moulds are not pathogenic, but some are however true parasites and produce a number of very common diseases. Their artificial cultivation is more difficult than the ordinary varieties.

Certain varieties of the common mucor has been reported pathogenic for man in that they have been found to produce eye and ear infections, also in a case of enteritis with secondary peritonitis. Autopsy of the latter showed also multiple abscesses of brain and lungs.

The aspergillus (*aspergillus fumigatus*, more frequent) is found more often pathogenic to birds, producing a pseudo tuberculosis. Such cases have also been reported in man.

Many varieties are found in plant diseases and indirectly may be of danger to man as when they form poisonous substances as in the infection of grain by *claviceps purpurea* (ergot poisoning), etc.

The more common pathogenic forms for man are:

Trichophyton (Ringworm Fungus).

1. *Tinea circinata* produces ringworm of the body.

2. *Tinea tonsurans* { produces ring-
3. *Tinea barbae* or { worm of the
 tinea sycosis { hairy parts.

According to Sabourand, there are two distinct types of the fungus, trichophyton causing ringworm in man.

(a) *Tinea microsporon*, with small spores, is the common fungus of *T. Tonsurans* of children, and its special seat of growth is in the substance of the hair. The spores are contained in a mycelium which is not visible, and appear piled up like zoogloea masses forming a dense sheath around the hair.

(b) *Tinea megalosporon*, with large spores, is essentially the fungus of ringworm of the beard and the smooth parts of the skin. The spores are always contained in distinct mycelium filaments, which may either be resistant when the hair is broken up or fragile and easily breaking up into spores. One-third of the cases of *T. Tonsurans* of children are due to trichophyton megalosporon. Cultivation is simple on acid glucose agar or gelatine.

Achorion Schoenleini (Favus). This fungus was discovered by Schoenleini in 1839. It attacks chiefly the hairy portion of the body of man and some domestic animals. It is communicated by contagion. Want of cleanliness is a predisposing factor. The disease is extremely chronic and very resistant to treatment.

In man, it is found most frequently on the scalp of persons in weak health, especially from phthisis, and in undernourished children upon the scalp. Other portions of the skin may also be involved. Pathologically, the disease represents the reaction of the tissues to the irritation caused by the growth of the fungus. The spores invade the hair follicles. The fungus grows in the epidermis, the density of the growth causes pressure on the parts below, lowering the vitality of the hair.

The disease first appears as a small sulphur-yellow disc (scutulum), pierced by a hair, which lesion is characteristic. It is readily cultivated on artificial media.

Kaposi has reported a case of confluent favus in which patients died with symptoms of severe gastrointestinal irritation. The presence of the fungus in the stomach and intestines was demonstrated at autopsy.

Microsporon furfur (Pityriasis Versicolor). This organism was discovered by Eichstedt in 1846, invades only the most superficial layers of the skin and produces the disease chiefly in those living under conditions of uncleanness, or among those who combine a tendency to profuse perspiration and uncleanness. The organism does not give rise to any considerable pathological changes in the skin or hair.

The organism shows preference for locations such as the chest, abdomen, back, and axillae, less frequently neck and arms, exceptionally it attacks the face. It appears as scattered spots of a color which varies from cream-coffee to reddish-brown.

Soor Fungus: Oidium Albicans (Thrush). This organism was described by Langenbeck in 1839. It produces a disease of the oral mucous membrane of infants during the early weeks of life. It occurs most frequently in children suffering from malnutrition. It has been found as a slight mycosis in the vagina of women and in rare cases attacks adults, especially those whose health has been undermined by diseases, such as diabetes, typhoid, etc. A few cases have been reported

in which the fungus was isolated from abscesses of the lung, spleen, kidney and brain. It can readily be cultivated in the ordinary media of either acid or alkaline reaction.

The *oidium albicans* appears both as a yeast and a mycelium, and therefore seems to occupy a position between the true moulds and the yeasts. It grows at times to long threads, and under certain conditions, almost exclusively, it will multiply by budding.

THE PATHOGENIC YEASTS.

(Blastomycetes).

These organisms have been of great importance in brewing and baking and recently have been reported to have caused infections in man and animals.

The position which the yeast occupies in systematic biology has as yet not been accurately determined. Their chief characteristic is in their method of reproduction by budding. Yeasts can at times develop short hyphae and in rare cases reproduce by segmentation.

The most important property of yeasts is that of producing alcoholic fermentation, and has been studied extensively along this line. The work of Pasteur and Hansen along these lines developed the fact that differences in the flavors and other qualities of beer, wine, etc., were dependent upon the particular species of yeast employed for the fermentation. The fermentative property is produced by an enzyme known as "zymase," which transforms sugar into ethyl alcohol. Various yeasts also produce other ferments which split higher carbohydrates (saccharose, maltose, starch) and prepare them for action of the zymase.

The yeasts employed in practice are spoken of as "culture yeasts" and those which act as disturbing factors in fermentation are called "wild yeasts"; the latter usually producing only a slight degree of fermentation. The culture yeast cell is oval or elliptical in shape, while the wild species are more often round or globular and known as "torula" forms. Sausage-shaped and thread forms are also found.

The individual cell is highly refractive and varies greatly in size even in those of the same species or the same culture. The cell contains a nucleus, which can be demonstrated by staining. During the process of budding the nucleus moves toward the periphery and divides, the limiting membrane of the cell ruptures or a protrusion develops (daughter cell), this rapidly increases in size and assumes the shape of the mother cell.

Spore formation takes place in the yeast, which is of importance in the continuation of the species and also for propagation. The nucleus divides into several fragments, each of which becomes the center of a new cell. These new cells (within the original cell) possess a firm membrane, a cell nucleus and a little dense protoplasm. As a rule one cell does not produce more than four spores, called "astrospores." The number of spores formed varies, however, but is constant for a species.

The Pathogenic forms are:

Saccharomyces Busse, isolated by Busse in 1894 from a woman's tibia. Autopsy showed broken down nodules on several bones, in the lungs and kidney.

Saccharomyces tumefaciens, isolated by Curtis in 1895 from multiple tumors on the hips and neck which resembled microscopically softened myxosarcoma.

This yeast is pathogenic for mice, rats and dogs. In generalized blastomycosis, the lung seems to be the seat of primary infection.

Cases described by Rixford and Gilchrist as coccidiosis (thought to be a protozoan disease) were unquestionably due to blastomycetes. Fontaine, Hasse and Mitchell reported a typical case of systematic blastomycosis. Lundogaard reported a case of ophthalmia due to a yeast. Tokishige reported an epidemic of ulcerous skin diseases among horses in Japan to be due to one of the saccharomyces. Kartulis described 100 cases of a skin affection occurring in the gluteal regions of men from which he isolated the ordinary saccharomyces cerevisiae. Kessler reported a skin

lesion in an infant as due to a blastomycete.

Attempts have been made to connect the development of cancerous growth with blastomycetes by reason of the similarity between the yeasts and the inclusions or so-called parasites of cancer and also by the fact that yeasts will, when injected into the animal body, produce tumor-like nodules. These masses are not tumors in a pathologic sense, but merely masses of yeast cells mixed with inflammatory tissue proliferation.

DISEASES OF UNKNOWN ETIOLOGY.

Measles. Many bacteria as well as supposed protozoan bodies have been described by various investigators as occurring on the mucous membrane or in the blood of those sick with the disease.

Home, in 1759, claimed to have produced measles of a modified and milder type by rubbing cotton swabs saturated with the blood of patients affected with measles on wounds made on the arms of other individuals. It is not certain that he produced the disease at all.

Positive results in experimental inoculation have been reported by Stewart (1799), Speranza (1822), Katowa (1842), and Nigirr (1850). These results are, however, not satisfactory.

Heckton, in 1905, produced the disease experimentally by injecting the blood of a measles patient, during the fourth day of the disease, into two students. Attempts at cultivation were negative, but the virus of measles will live for at least 24 hours when mixed with acetic broth.

Scarlet Fever is an acute, febrile, highly infectious disease characterized by a diffuse punctate erythematous skin eruption accompanied by catarrhal, croupous or gangrenous inflammation of the upper respiratory tract and by manifestations of general systemic infection. Both streptococci and protozoa have been described as the etiological factor in the disease.

Crooke in 1885 demonstrated streptococci in the cadavers of scarlet fever victims.

Babinsky and Sommerfield in 1900 reported the presence of streptococci in the heart's blood of eight rapidly fatal cases of scarlet fever.

Mallory in 1904 described protozoan bodies found in the skin of four scarletina cases. The bodies described and found by him between the epithelial cells were small and not unlike the plasmodium of malaria. They stain easily by methylene blue.

The bodies are still under investigation. Field and others have failed to demonstrate. The streptococci are, however, certainly present, but are considered secondary invaders, and by reason of this fact Moser adopted the use of antistreptococcic serum and claims exceptional results.

Typhus Fever. This is an infectious disease of a five-day or more incubation period and characterized by high temperature and a petechial rash. The etiological factor has not been definitely determined.

Nicolle in 1909 transmitted the disease to the chimpanzee, and from this to the macacus with typical eruption in each case. He was not able to transmit the disease from monkey to monkey.

Anderson and Goldberger in 1909 transmitted the typhus fever of Mexico (kabardillo) directly from the human being to the macacus and capuchin.

Ricketts and Walker in 1910 from researches came to the following conclusions:

1. *M. rhesus* can be infected by injecting the blood of man during the 8 to 10-day period of the fever.
2. It could not be transmitted from monkey to monkey.
3. The disease may produce so mild symptoms in the monkey as to be unrecognized clinically, vaccination results.
4. Immunity test is proof of previous occurrence or nonoccurrence of the disease within a period of one month.
5. It is transmitted to the monkey by the bite of the louse.

6. A monkey was infected with typhus by introducing feces and abdominal contents of infected lice into small incisions.
7. The blood of patients taken from the 7th to 12th day and stained (Giemsa) will show bacilli of the hemorrhagic septicaemic group morphology.
8. No cultures could be obtained, but fresh preparations showed forms like those above without motility.
9. Dejecta of lice were examined and the organism found in the infected lice and occasionally in noninfected ones.

Plotz has recently reported the cultivation of a gram positive pleomorphic anaerobic organism from the blood of cases of Brillo disease and also from typhus cases. Complement fixation was obtained when this organism was used as antigen.

Smallpox, or variola, is an acute infectious disease characterized by an epidemic eruption of macules, vesicles and pustules, which upon healing produce cicatrices of varying extent and depth.

The disease was first described by Phozes in the tenth century. It may have developed first in certain regions of Asia and central Africa.

Severe epidemics have swept China and Eastern countries many centuries before Christ, also Europe, especially at the time of the Crusades.

The disease was widespread when Jenner (1798) showed conclusively that vaccination with cowpox afforded protection.

The etiological factor is as yet not determined. Streptococci, often found in the vesicles and pustules and contribute materially to the fatal outcome of the disease, are secondary invaders.

Guarneiri (1892) named certain inclusions present in the epithelial cells of smallpox lesions in a rabbit's cornea. "Cytorrhycles variola," and believed them to be protozoa.

Councilman believed the bodies (vaccine bodies) to be protozoa, and describes two cycles in its development, one intracellular and the other intranu-

clear, and that intranuclear infection occurs only in smallpox.

Calkins, working with Councilman, also believes them to be protozoa of the class rhizopoda.

Ewing admits that the vaccine bodies are probably specific for variola, but calls attention to inclusions found in diphtheria, measles, glanders and other infectious processes which cannot be considered as etiological factors in these diseases. He believes that all forms so far described are degeneration products, some specific, others not. The similarity of cowpox (vaccinia) and small pox has been the subject of controversy. Many observers claim that although related to each other they are essentially different.

Others maintain, and this seems to be the prevailing opinion, that cowpox or vaccinia, when inoculated into man, represents the altered and attenuated variety of variola. It has been claimed that cowpox was originally transmitted to cattle by human beings affected with smallpox. The immunity caused by successful vaccination is not permanent and varies in its duration in different individuals. Although immunity may last for 10 to 15 years it is well to be vaccinated every year, if exposed to the disease. If the vaccination is unnecessary it will not be successful. (See smallpox vaccine.)

Rabies (Hydrophobia) is an acute infectious disease of mammals, dependent upon its specific virus and communicated to susceptible animals by the saliva of an infected animal coming in contact with a broken surface, usually through a bite. No bacteria have been discovered that are considered as factors in the disease. In 1903, Negri described peculiar structures which he observed in the cell of the central nervous system of rabid animals, which he claims are not only specific for hydrophobia but are probably animal parasites and cause the disease. His later studies confirm his previous work and, so far as the diagnostic value of these bodies is concerned, he has been corroborated by numerous investigators.

Williams in 1906, was convinced that these cell inclusions were animal organisms and called attention to the similarity between their structures and that of the rhizopoda. He gave them the name *Neuroryctes Hydrophobia*.

It is not possible, at the present time, to decide absolutely whether or not the Negri bodies should be regarded as parasites or specific degeneration products.

The virus of rabies has been shown to be partially filterable through coarse Berkefeld filters. The retained portion is always more virulent than the filtrate. This would seem to indicate that there are some forms just within the limit of visibility and others larger which correspond with what we know of the variation in size of the Negri bodies.

The largest forms of Negri bodies are about 18 microns and the smallest about 0.5 micron. They are round, oval, oblong, triangular or ameboid. They show a hyaline-like cytoplasm with an entire margin containing one or more chromatin bodies which have a more or less complicated and regular arrangement.

The demonstration of Negri bodies in tissues is carried out by procuring a small piece of tissue from the cerebellum or from the center of the hippocampus major and fixing it for 12 hours in Zenker's fluid. It is then washed in water and dehydrated in graded alcohol, embedded in paraffin and sectioned. The sections are attached to slides and placed in the following solution from 12 to 24 hours:

Methylene-blue (Grueber 00)	one
per cent.....	35 cc.
Eosin (Gruebler BA)	one per cent
.....	35 cc.
Distilled water.....	100 cc.

Then differentiate in:

Absolute alcohol.....	30 cc.
Sodium hydrate, 1 per cent in absolute alcohol.....	5 cc.

Allow them to remain for about five minutes, wash in absolute alcohol then place in water, then in water slightly acidified with acetic acid. Dehydrate in absolute alcohol, clear in xylol and examine.

The nerve cells are stained pale blue and in their cytoplasm close to the nucleus or near the axis-cylinder process are seen oval bodies stained a deep pink. They show a more deeply stained periphery than the interior which often contains small vacuole-like bodies. There may be one, three or four in a single cell.

The Negri bodies may be rapidly demonstrated in smears of brain tissues for diagnostic purposes as follows:

A small pin head sized piece of brain tissue taken from the cerebellum or the center of the hippocampus major is placed on one end of the slide under a cover glass and then gently squeezed until the tissue is flattened out into a thin layer. The glass cover is then gently slipped across the slide so as to smear the brain tissue along the entire surface. These smears are fixed in methyl alcohol and stained by the Giemsa method. The bodies are stained light blue in contrast to the darker and more violet cell bodies.

The smears may also be stained by van Gieson's stain as follows:

Fix the smears in methyl alcohol, wash in water, cover with the fresh prepared stain, steam, rinse in water and dry.

Distilled water.....	10 cc.
Saturated alcoholic solution of rosanalin violet	2 drops.
Saturated aqueous solution of methylene-blue diluted one-half	2 drops.

The Negri bodies stain magenta; their contained granules, blue; the air cells, blue; and the red blood cells, yellow.

Or by the Williams and Lowden modification:

Distilled water.....	10 cc.
Saturated alcoholic solution of basic-fuchsin.....	3 drops.
Loeffler's alkaline methylene-blue	2 cc.

The bodies assume a brilliant hue and contain in their interior darkly stained irregular particles, probably chromatin bodies.

All of the work should be controlled by careful animal inoculation. The work of the smear method in diagnosis has

been summarized by Parke and Williams as follows:

1. Negri bodies demonstrated, diagnosis rabies.
2. Negri bodies not demonstrated in fresh brains, very probably not rabies.
3. Negri bodies not demonstrated in decomposing brains, uncertain.
4. Suspicious bodies in fresh brains, probably rabies. (See rabic vaccine.)

Whooping Cough. (See Bordet and Gengou Bacillus.)

Bordet and Gengou in 1906, described a bacillus which they consider the specific organism because they obtain with it the complement fixation reaction.

Woolstein, 1909, was not able to corroborate their work. This bacillus differs slightly from a bacillus which differed only slightly from the bacillus of influenza that had been detected by Jockman, Krause and Woolstein in practically all cases of whooping cough during the acute stages.

Mumps. Diplococci had been considered as possibly being the exciting organism.

Noma. A streptothrix pseudo diphtheria bacillus, diphtheria bacilli are the organisms most usually present in cases of noma but it is, as yet, undecided whether the disease is due to one or to several microorganisms. A special predisposition of the tissues is necessary.

Articular rheumatism. (See Poynton and Paine diplococcus). Most bacteriologists believe the exciting factor has not yet been identified. Streptococci have been of all bacteria most frequently found in the search for the etiological factor in the synovial fluid, blood vegetation of heart valves, and in exudates on tonsils, etc.

The streptococci and the other cocci found are probably important secondary infections.

Beriberi. Both bacteria and protozoon microorganisms have been considered as exciting factors but nothing definite has been proven.

Pellagra. Some investigators believe this to be due to a microörganism, while others believe it to be an intoxication similar to that of ergot poisoning.

THE ULTRA MICROSCOPIC ORGANISMS.

Infective material from a number of infectious diseases may with certain precautions be passed through stone filters of varying degrees of porosity and still reproduce the disease with all its characteristic symptoms when inoculated into susceptible animals. Microscopic examination of the filtrate, except in one or two diseases, does not show the faintest sign of particulate matter.

The precautions necessary in such filtration are:

1. A perfect filter, which will absolutely retain all known bacteria, allowing none to pass into the filtrate.
2. The filtration must be completed within a moderate time by reason of the fact that bacteria may, in a media which contains a certain amount of albuminous material, grow through the filter.
3. The material to be filtered should be greatly diluted and first passed through filter paper so as to avoid the clogging action of extraneous material.

If, with these precautions, the filtrate is pathogenic, ascertain whether the symptoms are due to the microörganisms and not to a toxin. This may be determined with almost absolute certainty by inoculating a series of animals successively with the filtrate obtained from a previously so inoculated animal.

Anterior Poliomyelitis. Landsteiner and Popper thought the virus belonged to the class of invisible protozoa. They were able to transmit the disease to apes. They made intraperitoneal inoculations with spinal cord and produced typical symptoms and lesions. They were unable to transmit the disease from ape to ape.

Flexner transmitted the disease from monkey to monkey by intracerebral inoculations. Landsteiner and Le-

vaditi transmitted the disease from monkey to monkey and found that the virus lived four days outside of the body. They found the virus in the salivary glands and suggested the moist or dry saliva as a source of contagion. Flexner transmitted the disease by means of inoculations into the blood or peritoneal cavity, also by subcutaneous inoculation, and found the virus to be filterable.

The virus has been shown to be preservable under glycerin for ten days and retains its virulence to from 7 to 11 days, when dried. The virus will remain active when frozen for a period of 40 days, but is extremely sensitive to heat, being destroyed by a temperature of 45° to 50° C. maintained for 30 minutes. Flexner and Noguchi placed small bits of an emulsion of the brain of monkeys dead of poliomyelitis under conditions similar to the cultivation of the *Treponema pallidum*. Noguchi found after five days that an opalescence appeared, which increased until the tenth day when sedimentation began. Microscopical examination by Giemsa's revealed small ovoid bodies arranged in pairs, short chains and masses. Similar bodies were later found in poliomyelitis tissue. Cultures were obtained from glycerinated virus, fresh virus and from filtered and infiltrated material. When these cultures were injected into monkeys, typical lesions and death were produced up to the eighteenth generation of cultivation on artificial media.

Foot and Mouth Disease. This is a highly infectious disease occurring chiefly in cattle, sheep and goats, more rarely in other domestic animals. It appears as a vesicular eruption upon the mucus membrane of the mouth and upon the delicate skin between the hoofs. Usually the disease is mild. The vesicles form small ulcers and pustules which gradually heal with a disappearance of systemic symptoms. The disease may be complicated by a gastrointestinal or pulmonary infection and may end in death. The disease is generally transmitted from animal to animal by means of the virus con-

tained in the vesicles. On rare occasions, the disease is transmitted to man, usually by direct contact or by drinking the milk of animals suffering from the disease. The course of the disease in man is usually very mild.

Loeffler and Frosch in 1898, diluted the contents of an unbroken vesicle with 20 to 40 times its volume of water, passed the solution through a Berkefeld filter and found that the filtrate remains infectious for some time. The virus of the disease is readily destroyed by heating to 60° C. Loeffler has actively immunized horses and cattle with greater doses of the virus obtained from vesicles and with the sera of such animals has produced passive immunity. One attack of foot and mouth disease protects against subsequent attacks. This immunity may last for years, but a case of recurrence within a single year has been reported.

Yellow Fever is an acute infectious disease which prevails endemically in the tropical countries with no characteristic lesion except jaundice and hemorrhage. Other lesions that exist are those common to toxemia.

Sanarelli, 1897, found the bacillus icteroid circulating in the blood and in the tissues of most yellow fever patients and it was thought by many to be the causative organism, but has been rejected. The U. S. Army Commission (Reed, Carroll, Agramonte and Lazear) established the fact that the disease was carried from one infected person to another through the agency to the mosquito. This Commission established the following facts:

1. Yellow fever is transmitted under natural conditions only by the bite of a mosquito (*Stegomyia calopus*) that at least 12 days before had fed upon the blood of the patient, sick with this disease during the first three days of his illness.
2. Yellow fever can be produced artificially by subcutaneous injection of blood of a person sick with this disease during the first three days of his illness.

3. Yellow fever is not conveyed by fomites.
 4. The bacillus icteroid has no causative relation to yellow fever.
- Although the specific parasite has not yet been discovered, the following facts have been brought out:
1. The causative agent requires two hosts for the completion of its cycle. (A mammal and arthropod.)
 2. There is a definite time between the bite of a mosquito and the infectivity of the blood (average 5 days) and a definite time that the blood remains infective (three days.)
 3. The blood after passing through the finest porcelain filters remains infective during these three days.
 4. The blood will lose its virulence in 48 hours when exposed to the air and at a temperature of 24° to 30° C. If protected from the air by oil, at the same temperature, it remains virulent for from 5 to 8 days. It becomes non-virulent if heated at 55° C. for five minutes.
 5. The bite of an infected mosquito does not become infectious until 12 days (at a temperature of 31° C.) after it has bitten the first patient.

The infective blood filtrates show nothing with dark field illumination, except small motile granules similar to those found in healthy persons.

The necessity for a second host and the long incubation time required before the host becomes infected, after biting a yellow fever patient, seem to point to a protozoan organism as a causative factor.

Dengue, Ashburn and Craig claim to have reproduced the disease in susceptible individuals along the lines of procedure employed in yellow fever. The intermediate host in natural infection they claim to be culex fatigans.

South African Horse Sickness occurs in warm weather, as a rule, and seems to be more common in animals which pass the night outside. The disease manifests itself by uneasiness, difficulty in breathing, and the appearance

of reddish froth from the mouth. The temperature rises in the daytime, but drops at night. Edematous swelling of the head and neck may appear in severe cases.

Contagious Pleuropneumonia of Cattle.

This disease does not affect other species. It appears as inflammation of the lungs and pleura with necrosis. Nocard and Roux have cultivated an organism in collodion sacs placed in the peritoneal cavity of a rabbit, using a mixture of serum and bouillon. After two weeks a very faint turbidity appears in the sacs, coincidentally the fluid becomes infected. The causative factor in this disease has been made to grow and produce disease in new animals and, as at the highest limit or present magnification, it is seen to consist of minute granules.

Rinderpest. A fatal European and African disease of cattle is characterized by inflammation of the intestinal mucous membrane. No organism can be seen.

Trachoma is a disease of the eye which is characterized by a progressive follicular inflammation of the conjunctiva followed by cicatrization.

Prowazek (1907) announced the discovery of small organisms the cause of the disease and named them **Chlamydozoa** and believes they occupy a place between bacteria and protozoa.

The organism is found only in the early acute cases.

Prowazek states that the organism grows in a characteristic manner in the conjunctival epithelial cells. It is so small that it cannot at first be seen, only the mantle can be demonstrated, which stains blue with Giemsa. The organism appears as a small red granule within the blue body. As the organisms increase in size and numbers the blue mantles disappear, leaving a mass of small, round, or slightly elongated red bodies.

Lipschutz points out the fact that chlamydozoa, although visible, pass through filters, and with Borrel claims to have discovered a similar organism in *Molluscum contagiosum*

of man and birds. He also believes that the Volpino's motile granules discovered in vaccinia by the ultramicroscope and his own bodies of rabies belong to the same class.

By reason of their round form he suggested the name "Strongyloplasmen."

SPIROCHETAE AND ALLIES.

The microörganism known as spirocheta (name introduced by Ehrenberg in 1838, who differentiated it from spirillum by its flexibility) are slender, undulating, cork-screw like threads which vary both structurally and culturally from bacteria. The organisms were formerly regarded as bacteria, belonging to the general group of the spirillum. Schaudinn, by a careful morphological study, claims that many of these forms are protozoa. Other observers have not agreed with him.

The reason for considering these organisms as protozoa are:

1. Their flexibility and the indication in many of longitudinal division and of undulating membrane.
2. The demonstration of forms intermediate between trypanosomes and the spirochetes (SP. balbianii).
3. The spirochetal forms of certain trypanosomes (TR. Noctuae.)

The reasons for favoring the bacterial nature of spirochetes are:

1. The rigidity of some forms, the lack of undulating membrane in most and of definite nuclei apparatus in all, the evidence of transverse division of all and of flagella arising from the blepharoplast in some.
2. The cultivation of certain forms are the SP. refringens and the SP. Obermeieri for many generations without development of trypanosome forms.

It is probable that the spirochetes and their allies occupy a position intermediate between the protozoa and bacteria.

Spirochetes of the Mouth.

Non-pathogenic forms commonly found in normal mouths are:

1. *Spirocheta buccalis* has three to ten irregular flat coils. No true cilia have been demonstrated. Some authorities claim for it an undulating membrane. It stains violet with Giemsa.
2. ***Spirocheta Dentium*** is much smaller than the *buccalis* and somewhat similar to the *pallidum* in staining qualities and fixity of its coils when in motion. It stains with Loeffler's flagella stain. A flagellum is present, but no undulating membrane or nuclear material has been demonstrated. Spirals are numbered from four to twenty. This organism has been cultivated.
3. A form which seems to occupy a position between the two mentioned above has been found in the mouth, but has less regular spirals.

Spirocheta refringens is an organism that is found in the mouth. It is also frequently associated with *trypomonas pallidum* in the various lesions of syphilis, with which it is probably a secondary invader. The irregular, wide, flat spirals number from three to fifteen and change their shape during motion. Stains easily and quickly with Giemsa.

Schaudinn stated that it possesses an undulating membrane. A terminal cilia has been demonstrated by Levaditi, who also cultivated the organism in collodion sacs in the peritoneal cavity of a rabbit.

***Spirocheta Vincenti* (Vincent's angina).**

Vincent's angina consists of an inflammatory lesion in the mouth, pharynx or throat, situated most frequently on the tonsils, beginning as an acute stomatitis, pharyngitis, or tonsillitis, which soon leads to the formation of the pseudo-membrane closely resembling that caused by the bacillus of diphtheria. This may be followed by distinct ulcers with a well defined margin and punched out appearance. The disease is usually mild, but occasionally moderate fever and systemic disturbances are noted. Vincent described the presence of two organism as causative agents of the disease; the one a large spindle-

shaped, or fusiform bacillus; the other, a spirocheta similar to the "middle form" found in the mouth. By reason of the fact that these two organisms were always found together, they were at first believed to represent two forms which lived in symbiosis. Tunnickliff believes that these two forms merely represent different stages of development of the same organism.

The bacilli vary in length, thick at the center, from which they taper gradually towards the ends, and end in blunt or sharp points. The organism is usually straight, though sometimes it may be slightly curved. They stain with the stronger aniline dyes and usually decolorize by Gram's. They stain more deeply near the end and show a banded or striped alternation of stain and unstained areas in the central bodies.

The spirilla are usually somewhat longer than the fusiform bacilli. Microorganisms, as staphylococci, streptococci and not infrequently diphtheria bacilli, usually accompany the microorganisms of Vincent's angina, and by reason of this fact it is impossible to decide that the fusiform bacilli and spirilla are primary etiological factors. Animal inoculation with these microorganisms has led to little results.

Various fusiform bacilli which morphologically are indistinguishable from those found in Vincent's angina may frequently be found from smears from the gums, from carious teeth, and occasionally mixed with microorganisms in the pus from old sinuses. Weaver and Tunnickliff have found spirilla and fusiform bacilli in great numbers present in a gangrenous disease of the gums and cheeks, called noma. Here again, it is uncertain whether the organisms are primarily the etiological factor in the disease or merely secondary invaders.

Spirocheta Obermeieri (relapsing fever).

This organism was discovered by Obermeier in 1873 in the blood of patients suffering from relapsing fever. The organisms are long, slender, flexible, spiral or wavy filaments with pointed ends, with from

four to ten or more undulations. Compared with the red blood cells among which they are seen, the organism may vary from one-half to ten times the diameter of a corpuscle. They stain somewhat faintly with watery solution of basic aniline dyes, and stain best by the Romanowsky method or its modification. They are negative to Gram. A terminal flagellum has been demonstrated by Novy. The organisms are found in the blood or blood organs and never in the secretions, and only during the fever and not in intermission. In the fresh preparation from the blood, they exhibit active movements accompanied by very rapid rotation in the long axis of the spiral filaments, or undulating movement. Their movements will be active for a considerable time if kept in blood serum in 0.6 per cent sodium chloride solution. They are killed quickly at 60° C. but will remain alive some time at 0° C. Many unsuccessful attempts at cultivation have been made. Novy finally succeeded in cultivating them in celloidin capsules placed in the peritoneal cavity of rats.

The disease has been produced in monkeys, rats and mice by inoculating them with the blood containing the spirochetes. In man the organism produces the following symptoms:

The microorganism was found in a large percentage of the cases examined, both in the cutaneous papules and in ulceration. He determined that no monkeys are susceptible to inoculation. The monkeys susceptible to inoculation with yaws do not become immune to syphilis, neither do those having syphilis become immune for yaws. Further specific differences between the two disease have been shown by the Bordet-Gengou reaction. By reason of the morphological similarity to the tryponema pallidum, it should probably be called *treponema pertenus*.

Spirocheta Gallinarum is an acute infectious disease occurring among chickens, chiefly in South America. It is caused by a spirocheta which, morphologically, is very similar to the spirocheta of Obermeirel. It is

easily demonstrated in the circulating blood by staining the blood with Giemsa's stain or by dilute carbo fuschin. The organism has lately been successfully cultivated by Noguchi under anaerobic conditions. The disease has been transmitted from animal to animal by subcutaneous injection of blood. Other birds are susceptible as well. Mammals have not been successfully inoculated. The disease is generally transmitted to the chicken by a species of tick which acts as an intermediate host and causes the infection by a bite. Active immunization may be carried out by the injection of infected blood in which the spirochetes have been killed. The serum of immune animals has a protective action upon birds. Sacharoff had previously reported an organism named spirochete anserina, which caused a disease in geese, principally in Russia and Northern Africa, which clinically and pathologically corresponds to the disease caused by the spirocheta gallinarum, and it is not impossible that these two organisms may be identical.

A rapid rise of temperature which remains high for five to seven days then drops by crisis. At the end of seven days, another rise of temperature is followed by an earlier crisis. There may be a second or third relapse. The organisms multiply rapidly in the blood from the beginning of the fever. They begin to disappear a short time before the crisis, and after the crisis it is nearly impossible to find them in the circulating blood. The disease is not often fatal. The mortality in different epidemics varies from ten to two per cent.

The mode of transmission of the disease is not clear, though infection probably occurs through the bite of blood sucking insects. In the African disease the transmission occurs through the intermediation of a tick (*Ornithodoros moubata*), which infects itself when sucking blood from an infected human being.

Recovery from an attack usually results in a more or less definite immunity. The individuals who have recovered have hyper immunized blood. Both

the active and passive immunity may last for months.

Spirocheta Duttoni. Dutton in 1905 showed the cause for African tick fever to be due to an organism that was morphologically very similar to the SP. Obermeieri. Novy and Frankel believed that this organism is another variety if not another species of the group.

The organism can be transferred to monkeys by the bites of young ticks at their first feeding, after hatching from infected parents. Dutton accidentally inoculated himself through a break in the skin while performing an autopsy upon an infected subject, and died from the disease.

Spirocheta Carteri was described by Carter in 1877 as causing relapsing fever in Bombay.

Spirocheta Pallida (Treponema Pallidum). Schaudinn working with Hoffman, in 1905, while investigating a number of primary syphilitic indurations and secondary large lymphnodes, discovered a spirochete and named it *spirocheta pallida*. He thought that the organism was the cause of the disease. Further study by him revealed a delicate flagellum at each end, but left the existence of the undulating membrane which he at first thought present in doubt, so he called it *tryponema pallidum*. Extensive studies of human syphilis and experimental syphilis of lower animals has since corroborated the work of Schaudinn and Hoffman. The organism is a very delicate structure, closely resembling the *spirocheta dentium* in morphology and staining reaction. The spirals number from four to twenty and are quite deep. The angle of the spiral turn is very short. There are anterior and flagella-like prolongations. On rare occasions double flagella appear at one end, which Schaudinn interprets as beginning longitudinal division. Alive the organism is not very refractive, hence seen with difficulty. Its characteristic movements are rotation on its long axis, quivering movements up and down the spirals, slight forward and backward motion and bending of the entire body. The organism stains

red by Giemsa's method, as does also the spirocheta dentium. Most other spirochetes stain blue. The organisms have been found constantly present in the primary and secondary lesions of all carefully investigated cases. The presence of the spirochetes in the blood has been demonstrated by van Bandi and Simanelli. In the tertiary lesions the organism is found less regularly than in the primary and secondary lesions. In congenital syphilis the organism has been found in the lungs, liver, spleen, pancreas, kidneys, and in isolated cases in the heart muscle.

The organism may be demonstrated in the living state by the hanging drop method, which is, however, difficult and uncertain. A better method is by means of dark field illumination. The material taken for examination should be straight from syphilitic lesions, and if not dilute enough for examination, it should be emulsified in a drop or two of human syphilitic fluid.

The organism cannot be stained with the weaker aniline dyes, therefore the special method recommended by Schaudinn and Hoffman is generally used.

1. Make smears, if possible, from the depth of the lesion and free as possible from blood.

2. Fix in methyl alcohol for ten to twenty minutes and dry.

3. Cover with a fresh solution of:

Distilled water.....10 cc.

Potassium carbonate, 1 to 1000
.....5 to 10 drops.

Giemsa's solution.10 to 12 drops.

Allowing the mixture to act for one to four hours, preferably in a moist chamber.

4. Wash in running water.

5. Blot and examine.

The organism is stained with a violet or reddish tint.

The organism may be demonstrated in tissues by the method of Levaditi.

1. Fresh tissue is cut into small pieces of two to four m.m. thickness.

2. Fix in ten per cent formula for 24 hours.

3. Wash in water.
4. Dehydrate in 96% alcohol for 24 hours.
5. Wash in water.
6. Place in a three per cent silver nitrate solution at a temperature of $37\frac{1}{2}^{\circ}$ C. and in the dark for three to five days.
7. Wash in water.
8. Place in a freshly prepared solution of:

Pyrogallie acid.....	2 to 4 grams
Formalin.....	5 cc.
Distilled water.....	100 cc.

 Allow to remain in this solution for 24 to 48 hours at room temperature.
9. Wash in water.
10. Dehydrate in graded alcohol.
11. Embed in paraffine and cut thin sections.
12. Examine without further staining or counter stain with Giemsa's solution or Hemotoxin.

Attempts at cultivation were at first unsuccessful. Later cultivations have been reported by Schereschewsky and Muehlens but did not succeed in carrying out Koch's postulates with the cultures they obtained.

Noguchi has successfully cultivated the spirochete as follows:

Into tubes (20 cm. high and 1.5 cm. wide) he placed 10 cc. of a serum water made of three parts of distilled water and one part of coarse sheep or rabbit serum. These were sterilized by the fractional method, after which a small piece of sterile rabbit kidney or testicle and a bit of testicle of syphilitic rabbit were placed in each tube.

The serum in the tubes was now covered with sterile paraffin oil and placed in an anaerobic jar at $33\frac{1}{2}^{\circ}$ C. for ten days, at which time, the spirocheta had developed greatly together with bacteria. He obtained pure cultures from these cultivations by allowing the spirochetes to grow through Berkefeld filters; also by what he considers a better method, preparing high tubes of three parts of very slightly alkaline or natural agar to which a piece of sterile tissue had been added. These tubes are inoculated from the impure cultures with a

long pipette. The spirocheta and bacteria grow close to the tissue and along the stab. At the end of ten days to two weeks, the spirocheta wander from the stab and appear as hazy colonies. The tubes are cut and the colonies are directly transplanted to other serum agar tissue tubes. Noguchi carried out Koch's postulates with syphilis.

So far as is known, syphilis in nature appears only in man. All experimental inoculations of animals were unsuccessful until Metchnikoff and Roux (1903) succeeded in transmitting the disease to a female chimpanzee. Klebs stated, 1879, that he had produced syphilis in monkeys by the inoculation of human virus. Since then Lazear has also successfully inoculated monkeys. Nicolle succeeded in inoculating the lower monkeys (*Macacus*) with syphilis. Attempts to transmit syphilis from the tertiary lesions have been unsuccessful. The organisms can be demonstrated both in primary lesion and in the secondary enlarged gland of the inoculated animal. Bertarelli produced an ulcerative lesion of syphilis by inoculating upon the cornea and into the anterior chamber of the eye, and later found the spirocheta within this situation. Syphilis generally remains localized in rabbits as well as in the lower monkeys. Parodin, in 1907, inoculated syphilis into the testicles of rabbits, and this method has proven to be the most simple in obtaining the spirocheta from lesions in man and indefinitely carried along by continuous transinoculation from one rabbit to another.

After the development of the primary lesion man is usually insusceptible to reinoculation during the active stage of the disease, but in some cases both man and monkey can be reinoculated. Reinoculation in the tertiary state produced precocious lesions of tertiary type, gumma and tubercles. Injections of large quantities of syphilitic serum into chimpanzee has failed to produce definite immunity.

For the Bordet-Gengou phenomena see Wasserman reaction under Complement Fixation.

Spirocheta Pertenuis (*Framboesia tropica*, yaws). Yaws, a disease resembling syphilis, occurs in tropical and subtropical countries, and Castellani in 1906 announced that he had found a spiroörganism which bore a close resemblance to the *spirocheta pallida*. He named it *spirocheta pertenuis*.

Spirocheta Phagedenis is an organism of probably a new species cultivated by Noguchi from the phagedemic lesions on human external genitals.

Spirocheta Macrodentium is believed by Noguchi to be identical with Vincent's *spirocheta*.

Spirocheta Microdentium cultivated by Noguchi from the tooth deposits in children.

Spirocheta Calligyrum cultivated by Noguchi from condylomata is probably a new species.

THE BACTERIOLOGY OF MILK.

The use of cow's milk as a food, especially for infants, has caused it to be closely studied.

Milk usually contains about 87% of water and about 13% of solids. Of the solids there is approximately 4% of fat; the remaining 9% is composed of about 5% lactose, about 3.3% protein (caseinogen 4 parts, albumen 1 part) and about .7% of ash (salts). There are in addition hydrolitic enzymes as galactase, a proleolytic enzyme and oxidase.

Milk is a favorable culture medium for the development of bacteria and therefore very well fitted to convey the germs of infectious diseases.

It is ordinarily impracticable to secure milk entirely free from bacteria. In the milk ducts and in the teats of even healthy cows a certain number of bacteria may be found, although within the udder milk is sterile.

If pyogenic or systemic disease of bacterial origin exists, the milk may be infected.

Certain forms of bacteria seem to develop within the milk cistern and within the larger milk ducts. The first milk drawn from the teats is generally loaded with bacteria, in the later milk they are comparatively few in number in comparison.

Usually milk drawn from the udder contains less than 100 bacteria per 1 c.c., although in some cows, seemingly normal, there may be large numbers.

There are changes taking place in milk, due to micro-organisms, which in a sense may be considered normal and may be divided into a stage of bacterial action, a development of lactic acid, a neutralization of lactic acid and a decomposition or putrefaction.

Incidental changes, brought about by bacteria, such as sweet curdling, ropy, soapy or color formation, may infrequently be met with.

The most important source of bacteria in milk is probably due to contamination of the milk from dust particles containing bacteria, which are dislodged from the hair and skin of the udder and sides of the cow during the process of milking. It is therefore necessary to have the animal carefully groomed and adjacent body surfaces thoroughly moistened in order that this source of contamination may be eliminated while milking. The organisms from this origin are largely of fecal origin.

Dust in the building in which cows are milked, from dusty fodder or bedding, is also a source of contamination, and are usually the *B. subtilis* and putrefactive types of bacteria.

The hands of milkers, unless carefully cleaned, will also afford an opportunity for milk infection. This contamination may more readily carry infection from the milkers to other individuals, than an infection from the cow itself to man.

Milking utensils may also prove a dangerous source of infection, in that imperfectly soldered joints may harbor innumerable bacteria. Utensils should be thoroughly scalded, or the entire vessel heated to the boiling point of water to destroy the organisms present.

Careless handling, such as allowing milk to stand in open cans or the use of unclean dippers, etc., or contaminated water used for rinsing milk vessels, is frequently a cause of contaminating clean milk.

The number of bacteria in fresh milk will decrease for a time, which indicates a germicidal action. The length of time

of bactericidal action differs with the number and kinds of bacteria and with the conditions under which the milk is kept. Arguments as to the reduction of bacteria are many. Some hold that reduction is due to agglutinating power of milk, so that in reality there is no actual reduction in bacterial number. Others argue that all bacteria gaining entrance to milk do not find favorable environment and die more rapidly than those which find environment suitable.

It would seem, however, that milk must contain a certain amount of bactericidal action by reason of germicidal substances contained in blood, which must be given off, at least in part, with the milk.

The number of leucocytes present in milk would also be a factor, as their phagocytic power would not be lost immediately with the milking.

The bactericidal property, however, can in no case completely sterilize milk, as the bactericidal action is specific, that is, certain bacteria are destroyed by it, while others are not affected.

The lactic acid organisms, present in milk, develop rapidly, particularly if milk is kept in a warm place. When these organisms develop 0.4% of lactic acid the milk will be decidedly sour in taste. When .75% to .80% acidity is reached, curdling of milk takes place.

The ordinary lactic acid bacteria will rarely produce more than a 1.25% acidity. The *Bact. bulgaricum* group of bacteria will, however, produce a much higher percentage of acidity.

Sour milk may be kept under anaerobic conditions for a long time without producing any change in its composition. If exposed to the air, however, certain molds (e.g., *Oidium lactis*) develop on the surface of the milk, using the lactic acid as food, oxidizing it to CO_2 and water. By reason of this the acidity of the milk is neutralized. Some of the acid may also be neutralized by the milk caseinogen.

When the excess milk acidity has been neutralized, the various putrefactive bacteria develop and the milk, particularly the caseinogen, rapidly decomposes.

Milk heavily inoculated with the *B. subtilis* group of organisms may not sour, but undergo sweet curdling instead. This is due to the overgrowth of the lactic acid organisms and the production of a rennet-like enzyme by the *subtilis* group. The curd is later more or less completely digested.

Certain organisms produce the so-called ropy milk by the formation of gums from carbohydrates and mucin-like substances from the proteins, while certain other organisms may produce red, yellow, blue and even black milk.

The undesirable flavors, sometimes produced in milk, characterized as soapy and bitter milks, are produced by bacteria.

The number of bacteria present in a given sample of milk depends upon the contamination taking place during the milking process, the time which elapses after the milking, the temperature at which the milk is held, the care in handling and the matter of non-pasteurization or pasteurization.

The temperature at which milk is held is very important. The acid-producing organisms and most other forms grow slowly if at all at low temperatures. Milk should therefore be cooled as soon as possible after drawing and kept at a low temperature so as to prevent the multiplication of bacteria. If this is carried out milk may be kept from souring several days.

If it is not quickly cooled and is kept at room temperature it may sour in less than 24 hours.

The number of bacteria may be greatly reduced by pasteurization.

Infections transmitted by milk.

The most important infections transmitted by milk are the diarrhoeas and dysenteries of infants. The intestinal tracts of infants seem particularly susceptible to infection of micro-organisms belonging to the enteriditis, paratyphoid and dysentery groups. The summer complaints of infants are, in large part, due to the use of milk containing these organisms. Wherever it is impossible to obtain an infection-free milk for infant feeding, pasteurization becomes necessary.

Typhoid fever epidemics have frequently been traced to an infection through the milk supply. This is also true for scarlet fever and diphtheria.

The use of tuberculous milk is the common cause of a number of cases of tuberculosis in children, the milk having been contaminated by the organism entering the milk within the udders of cows or, what is more likely, by contamination through the feces of animals sick with the disease.

Milk should come from herds that have been tested by tuberculin, and from which all of the tuberculous animals have been removed.

Anthrax, foot and mouth disease, and malta fever have infrequently been transmitted through milk.

Milk Analyses.

1. Plate various dilutions of milk on nutrient agar (+ 10 reaction).
2. Incubate at 37° C. for 48 hours, or at 22° C. for 5 days.

Milk properly drawn will not contain more than 500 to 1000 bacteria per 1 c.c. Milk as sold in cities is from 36 to 48 hours or over old before use, contains many times the above number.

Good milk may contain about 1,000,000 bacteria per 1 c.c., or even more when it begins to sour, so that it is evident that numbers alone are of little moment except that they indicate the care used in milking and delivery to the consumers.

Certain cities have classified milk into uninspected milk, inspected milk, pasteurized milk and certified milk.

Uninspected milk has no sanitary control.

Inspected milk is a milk which comes from cows tuberculin tested, and which is drawn and cared for under sanitary conditions.

Pasteurized milk is a milk which has been heated for a short period of time at a temperature considerably below the boiling point, and then followed by a rapid chilling. Its object is the destruction of harmful bacteria and their products.

The two methods of pasteurization are:

- (1) The "holder process" in which the milk is heated to 60–65° C. and

held at this temperature for about one-half hour. It is then cooled rapidly and bottled.

- (2) The "flash or continuous process" in which the milk is heated to 80–85° C. and held at this temperature for 30 seconds to one minute. It is then cooled and kept at a low temperature until distributed.

The holder method is held to be the most efficient, as it destroys the larger percentage of bacteria.

Certified milk is now a milk obtained from animals free from contagious or infectious disease; attendants must be in good health; stables must be sanitary, well lighted, and free from dust; milking vessels must be sterile, and every precaution must be used to prevent the entrance of bacteria to the milk. After milking it must be quickly cooled, sealed in bottles, and kept cold until delivery. In most cities where certified milk is inspected it must not contain more than 10,000 bacteria per 1 c.c.

THE BACTERIOLOGY OF WATER.

All natural waters contain micro-organisms, which gain entrance from many sources. The vapors arising from the sea or land contain no organisms, but as soon as precipitation takes place, the organisms enter the water from the air and soil.

Certain organisms, because of their ability to find sufficient nutriment for life and growth in water, may be spoken of as belonging to the "**water flora.**" Some bacteria, found in water, flourish only during rain and flood seasons, while other bacteria, such as intestinal organisms, survive for a short period only.

The organisms found in water may be divided into:

1. **The Natural Water Bacteria**, which are frequently numerous and generally harmless to man. Certain species will predominate at one season and disappear at another. Some bacteria contained in water have, by reason of their biochemical properties, been divided into:

- (a) **A bacillus fluorescence liquefaciens group**, recognized by the green fluorescence of the colonies and liquefaction of gelatine, is more frequently found in water than in any other form.
- (b) **A bacillus fluorescence non-liquefaciens group**, produce the characteristic fluorescence, but do not liquefy gelatine. are very abundant in river water and are represented by the *B. f. longus*, *B. f. tennis*, *B. f. aureus* and *B. f. crassus*.
- (c) **A group of liquefying and milk acidifying bacilli**. These are common to certain seasons. Some are soil organisms, some are related to the proteus group, others are the *B. liquefaciens*, *B. punctatus* and *B. circulans*.
- (d) **A chromogenic bacilli group** such as *B. prodigiosus*, *B. ruber*, *B. indicus*, *B. rubescens*, *B. rubefaciens*, *B. aquatilis*, *B. ochraceus*, *B. aurantiacus*, *B. fulvus*, etc., are often present in water.

At certain times, in river and brook waters, violet organisms as *B. violaceus* or *B. janthinus*, *B. lividus*, *B. amethystinus* and *B. coerulescens* are found.

- (e) **A chromogenic cocci group**. *Sarcina lutea* is the most common species, though this group is not numerous in water.

- (f) **A non-chromogenic cocci group**, such as the non-liquefying *M. candidans*, *M. nivalis*, *M. aquatilis*, and a liquefying type as the *M. coronatus*.

2. **Soil bacteria from surface washing**. Numerous soil organisms are found in natural waters during floods and after rains. Certain species of these organisms may remain in the water for a long time.

The most common organisms of this group are the *B. mycoides*, *B. subtilis*, *B. megatherium*, *B. mesentericus* (*vulgatus*, *fuscus* and *ruber*).

The colonies of these organisms are characteristic rhizoid, liquefying gelatine, and produce spores. One of the thread bacteria (*cladotrix dichotoma*) may also be present. It is frequently found in both fresh and stagnant water and in most soils.

For the isolation of these organisms, beef peptone gelatine is used. When other media are used a different flora, such as the nitroifying organisms, yellow chromogens, etc., appear.

3. **Intestinal Bacteria**, usually of sewage origin.

- (a) **Protein group.** The *B. vulgaris*, *B. zenkeri*, *B. mirabilis*, *B. zopfi*, *B. cloacæ* and the sewage proteins of Houston.

These organisms are very abundant in sewage, but are not present in very large numbers in contaminated water. This group of organisms are mobile, liquefy gelatine, produce gas in dextrose and saccharose broth (sometimes a little in lactose), reduce nitrates, coagulate milk, produce indol and impart a fecal odor to the media.

- (b) **Sewage streptococci.** The streptococci in water is indicative of recent sewage contamination. They die quickly outside of the body. By their action on the various sugars an equine, human and bovine type may be differentiated, which may be used as indicative of recent contamination from street washings, human excreta or cultivated fields.

- (c) ***B. enteritidis sporogenes***, though usually present in the intestinal tract of man, cannot be considered as an indicator of excretal pollution by reason of its presence in dust, food stuffs, etc., and the resistance of its spores.

- (d) ***B. Coli*.** The bacillus coli is accepted as the bacterial indicator of sewage pollution of water.

- (e) ***Bact. lactis ærogenes***, next to *B. coli*, may be regarded as an indicator of sewage pollution of water.

- (f) ***B. typhosus***, reported to have been isolated from water in a very few instances, will live in pure water from 8 to 10 days. When exposed to the action of sewage bacteria, it will live for from 5 to 6 days.

- (g) ***Msp. comma*.** The spirillum of Asiatic cholera is an intestinal organism and spreads the disease largely through water. It has been frequently isolated from infected waters.

The number of bacteria in water.

The bacterial purity of natural waters depends upon the source from which the waters are derived, together with the special and local condition in relation to contamination.

Rain. The number of bacteria present in rain water depends upon the month of the year and the dryness of the air. When there is considerable dust in the air, the first rain will be very rich in bacteria, but during the latter hours of prolonged rain the water may be comparatively sterile.

The rain in densely inhabited cities always contains more bacteria (averaging about 19 per cc.) than the rain falling on open farm land or upland pastures, in which the number of bacteria will average about 4.3 per cc.

Snow. The results from snow fall are similar to those from rain, except that the number of bacteria present per cc. is larger; 334 to 463 bacteria per 1 cc. of snow water has been recorded, while Binot did not find a single micro-organism present in 8 cc. of water from mountain-top snow.

Hail Stones. The number of bacteria obtained from hail stones varies from 628 to 21,000 per cc. by reason of surface water being carried by storms.

Well Water. Deep well water ordinarily contains but few organisms. Usually less than 50 per cc. on gelatin at 20° C. and about 5 per cc. on agar plates.

Shallow well water's bacterial content varies with the amount of rain fall, even though they are well located and constructed. The water in polluted wells may contain enormous numbers of organisms; 20,000 bacteria per cc. on gelatin has been reported.

Spring Water corresponds in bacterial content to that of deep wells.

Upland Surface Water contains but few bacteria if draining from barren land. Cultivation and habitation may change this considerably.

Pure waters contain from 50 to 300 bacteria per 1 cc. when grown on gelatin and from 1 to 10 on agar.

River Water. The bacterial content of river water is influenced by sewage contamination, temperature, rain fall, vegetable debris, etc.

Lake Water is generally much purer than the waters of rivers. The bacterial content near the shore is greater than further out by reason of the influence of habitation.

Sea Water near the shore and in the neighborhood of seaports may contain a large number of bacteria. In the water remote from the coast there are few bacteria.

The number of bacteria in natural waters is influenced by:

1. **Temperature.** A low temperature decreases the parasitic types, but the number of other bacteria present during the hot summer months is generally somewhat less than during the cooler months.

Water should be examined for its bacterial content immediately after collection, as there is usually a reduction in the number during the first few hours, to be followed later by a large increase.

The samples collected for analysis should be kept cool, although very polluted waters show a marked decrease of intestinal types if the sample is kept cool.

Light, although germicidal, does not influence the number of bacteria in water, probably by reason of the water's turbidity and the speed of the current. The greatest germicidal effect of sunlight is produced in shallow, clear and slowly moving water. Direct light is not efficient as a purifier of water.

Food Supply. In water containing a large amount of organic matter the number of bacteria is greatly in excess of that in which there is but little of such material. Sewage water is rich in organic matter and therefore contains great numbers of bacteria. The number of bacteria present in a given water is therefore proportionate to the diminution of organic material. Self purification

of streams is dependent mainly upon the causes producing insufficiency or unsuitability of the bacteria's food supply.

Oxidation. The oxygen absorbed on the surface of waters in rapids, falls and tidal rivers can be considered as a very minor agent in the purification of water.

Low Plants and Animals as algæ, river plants and numerous protozoa reduce the organic matter of water and thereby reduce the food supply of bacteria. The chemical products of higher forms are injurious to bacteria and many bacteria are ingested by the unicellular animals.

Dilution. Polluted water flowing into larger bodies of pure water, as into a river or lake, is at once diluted, thus diminishing the bacteria's food supply, likewise also diffusing the bacteria through a greater volume of water; the greater the dilution the fewer sewage bacteria will be found.

Sedimentation. The suspended matter of still water tends to sediment, and this in itself brings about its purification.

Water Analyses.

The improbability of getting typhoid bacilli from suspected water, except under unusually favorable conditions, caused a return to the estimation of the number of intestinal bacteria.

It is known that the group of colon bacilli have a somewhat longer existence than the typhoid bacilli, and as the colon bacilli come chiefly or wholly from the intestinal passages of men and animals, it is fair to assume that typhoid bacilli could not occur without the presence of the colon bacillus, except in rare cases, as, for example, pollution with urine only. The latter could of course occur abundantly without the typhoid bacillus.

During the past few years the attention of sanitarians has been seriously devoted to the interpretation of the presence of smaller or larger numbers of colon bacilli in water, until at present upon the quantitative analyses (measuring within certain limits, de-

composing organic matter) and the colon test (indicating more specifically that pollution derived from intestinal discharges of man or animals) the bacteriological analyses of water is based.

The determination of the number of bacteria is also of value.

Technique. Utmost care is necessary to get reliable results. A speck of dust, a contaminated dish, a delay of a few hours, an improperly sterilized agar or gelatin, a too high or too low temperature, may introduce an error or variation in results which would make a reliable test impossible.

In the collection of sample:—

1. Utmost carefulness in collection is necessary.
2. An immediate test is essential as bacteria readily increase or decrease in number after collection.

Frankland records a case (well water sample) of water sample, kept for 3 days at a moderate temperature, in which the bacteria increased from 7 to 495,000.

Jordan reports a case of sample water in which the bacteria decreased in 48 hours from 535,000 to 54,000.

Park and Williams, of New York City, record a case in a sample from Croton river in which B. Colon present increased from 10 to 100 per c.c., during 24 hours.

3. It is better to make cultures in the open field or in a house, rather than to wait 12 hours for the conveniences and advantage of a laboratory.
4. If sent to the laboratory, water should be kept at about 5°C. (41° F.) during transit.

Quantity of water to be used in tests.

1. It is of great importance to add proper amounts of water to the broth in the fermentation tubes and in the media for planting. Usually 1 c.c. and 0.01 c.c. are added to the fermentation tubes and 10 c.c. of melted nutrient agar or gelatin.
2. If possible always make duplicate tests.
3. When necessary to know whether colon is present in larger amounts than 1 c.c., quantities as large as

10 c.c. or 100 c.c. can be added to bouillon, and then after a few hours 1 c.c. are added to fermentation tubes.

4. Less than 20 colonies and more than 200 on a plate give inaccurate counts, the smaller number being too few to judge an average and the larger number interfere with each others growth.

When as many as 10,000 colonies develop in the agar contained in one plate, it will be found that there will develop in a second plate containing but 1-10 the amount of water from 20 to 50% as many colonies. This shows that crowding of the colonies had prevented the growth of all but 1-5 to $\frac{1}{2}$ of them.

5. The chemical composition of the medium affects the results of the analyses.

Nutrient agar of a 1.5% acid reaction gives slightly lower counts than gelatin, but on account of its convenience in summer and its greater uniformity, it is more generally used for routine work.

6. The American Public Health Association has adopted a standard reaction of 1% acidity which is the average optimum for water bacteria.

Only a certain proportion of bacteria develop and all we can ask is that our count represents fairly the quick growing sewage forms.

7. The temperature is very important. Plate cultures, as a rule, are grown at 20°C.-21°C. for days, and at incubator temperature (37.5°C.) for from 24 to 48 hours.

Some bacteria do not develop in 4 days, but these are neglected.

The number of bacteria growing at room temperature is usually much greater than those growing at 37.5°C.

As all the intestinal groups of bacteria grow at body temperature, while many of the water types do not, some investigators believe it important to develop the bacteria at both temperatures so as to compare the results. (Advantage in coli tests).

8. In making litmus lactose agar plates, the colon, if present, will take on a red color in the blue field. If many coli are present the whole medium becomes red by reason of the acidity. Later, at 48 hours or so, by reason of an alkali being produced by the formation of NH_3 , the blue color may return.

Significance of Coli Bacilli in Water.

The colon test has been applied with satisfaction and confidence in the examination of water, shell fish, and other articles of food by many authorities, while other authorities have denied its value.

Bacteriologists have found bacilli resembling certain members of the colon group in apparently unpolluted well water.

The discovery that animals have colon bacilli identical, in the usual characteristics studied, with those of man has complicated matters.

A fresh hill side stream may be loaded with colon bacilli from the washings of horse or cow manure used as fertilizer in the soil of the field through which the stream runs, or the stream may be polluted by a stray cow or horse. Swine, hens, birds etc., may contaminate in unsuspected ways.

The number of colon bacilli, rather than their presence, in any body of surface water is therefore of importance.

In well and spring water the presence of the colon bacilli indicates contamination. The absence of the colon bacillus in water proves it harmless so far as bacteriology can prove it.

When the colon is present in numbers that may enable one to isolate it from 1 c.c. quantities in a series of tests, it is reasonable proof of animal or human contamination and the conditions should be investigated. 10 colon in 1 c.c. indicates serious contamination.

Surface water from inhabited regions will always contain numerous colon bacilli after a heavy rainstorm or shower.

The washing from roads and cultivated fields contain necessarily large numbers of colon bacilli.

Wilson reports that in only two out of 58 samples of presumably non-pol-

lute waters did he recover colon bacilli in 1 c.c. samples; even in 21 stagnant pools he only found colon bacilli in 5 of the 1c.c. samples.

The experience of all those who have studied the subject practically, is that in delicacy the colon test surpasses chemical analysis; in constancy and definiteness it also excels the quantitative bacterial count. All tests must, however, be supplemented by inspection.

Analyses

1st Method.

1. Plate 1 c.c. of water in each of 2 or 3 Petri dishes containing Hess's agar. Incubate for from 24 to 48 hours at 37.5°C., at end of which time, count the colonies appearing on the plate.
2. Plate 1 c.c. of water in each of 2 or 3 Petri dishes containing gelatin. Incubate for from 48 to 96 hours at 20-21°C., at end of which time, count the colonies appearing on the plate; make note of the number of liquefiers.
3. Place 1 c.c. of water in each of 10 fermentation tubes containing dextrose bouillon. Incubate at 37.5°C. for 48 hours. Note the quantity of gas produced, if any, at the end of 24 hours, also at the end of 48 hours. Determine the ratio of CO₂ to H.

Fermentation with the proper gas ratio in but one tube would suggest that 1 colon bacillus is present in 10 c.c. of water, etc.

Make report on number of bacteria present in 1 c.c. of water grown on agar.

Make report on number of bacteria present in 1 c.c. of water grown on agar.

Make report on number of bacteria present in 1 c.c. of water grown on gelatin.

Make report on number of liquefiers present in 1 c.c. of water grown on gelatin.

Make report on number of colon bacilli present in 1 c.c. of water grown in dextrose bouillon.

2nd Method.

1. Plate 1 c.c. of water in each of 2 or 3 Petri dishes containing litmus lactose agar and incubate at 37.5°C.

for from 24 to 48 hours, at end of which time note number of red colonies, and transfer these to each of the necessary number of fermentation tubes containing 1% dextrose bouillon. Incubate the tubes for 24 hours. If gas is not present, the red colonies are not colon bacilli.

If gas is present, test gas ratio, then apply Riva's test for colon 1, 2 and 3.

Riva Test No. 1.

Boil in a test-tube about 5 c.c. of media from the fermentation tubes, with about 3 c.c. of a 10% solution of Na OH.

No change in color=colon bacillus.

Change in color to a pink=not colon, but ordinary saccharolyte.

Riva Test No. 2.

Depends upon the ability of colon to exhaust all sugar in a 1% dextrose bouillon in 24 hours. Sugar change as a matter of fact ceases at the end of the 18th hour.

Determine the presence or absence of sugar in the media of fermentation tube culture by boiling a small quantity in a test-tube containing about 5 c.c. of Fehling's solution. A reduction of the copper by the sugar present, changing blue color to deep yellow to red ppt., indicates colon bacillus.

No reduction of the copper, if no sugar is present, consequently no change in blue color=not colon.

Riva Test No. 3.

Add to about 5 c.c. of fermentation tube culture, contained in a test-tube, about 3 c.c. of a 50% solution of H_2SO_4 then add 2 or 3 c.c. of a 10% solution of NaOH.

A pink to red contact ring=Indol reaction=colon.

3rd Method.

Vide—1, 2 and 3 of 1st method. Then apply Riva's 1, 2 and 3 tests to the fermentation tube cultures showing gas formation.

4th Method.

Quantitative Examination.

- A. 1. Plate 1 c.c., 0.5 c.c., 0.3 c.c. and 0.2 c.c. of water in agar.
2. Plate 0.5 c.c. and 0.1 c.c. of water in agar.
3. Plate a controle agar.

4. Label each plate with the number of the sample, the quantity of water contained and the date.
5. Incubate at 37.5° C.
- B.
 1. Place 9.9 c.c. sterile distilled water in a sterile capsule.
 2. Add 0.1 c.c. of the water sample to 9.9 c.c. of water in the capsule. This will give a dilution of 1 in 100.
 3. Plate 0.5 c.c., 0.3 c.c. and 0.2 c.c. of diluted water in gelatin.
 4. Label each plate with the quantity of water it contains—that is, 0.005 c.c., 0.003 c.c., and 0.002 c.c.
 5. Plate 0.5 c.c., 0.3 c.c. and 0.2 c.c. of water sample in gelatin.
 6. Plate a controle.
 7. Label each plate with the quantity of water it contains.
 8. Incubate at 20° C.
- C.
 1. Plate 0.5 c.c., 0.3 c.c. and 0.2 c.c. of water sample in wort gelatin.
 2. Label the plates and incubate at 20° C.
- D.
 1. After 48 hours incubation, count and record the number of colonies that developed upon the various plates.
 2. Replace the gelatin and the wort plates in the incubator; observe a gain at 3, 4 and 5 days.
 3. Calculate and record the number of organisms present per c.c. of the original water from the average of the six gelatine plates at the latest date possible up to seven days. The presence of liquefying bacteria may render the calculation necessary at an earlier date, hence the importance of daily observations.

Qualitative Examination.

In routine examination of water, the qualitative examination of water is usually limited to a search for *B. Colli* and its allies, streptococci and some observers insist on a search for the *B. enteritidis sporogenes*. The last organism is relatively scarce in water, therefore, the collection of a large quantity of water is usually necessary.

During epidemics or the examination of new and unknown waters, the colityphoid group are to be searched for and on occasion the presence or ab-

sence of vibriocholera, *B. anthracis* or *B. tetani* may need to be determined.

When pathogenic or excremental bacteria are present in water, their number are few and it is, therefore, necessary to adopt either the enrichment or the concentration method of examination.

A. Enrichment Method. The harmless non-pathogenic bacteria are destroyed or their growth inhibited, while the growth of the parasitic bacteria are encouraged by arranging the environment as to reaction of media, incubation temperature and atmosphere so as to favor the growth of the pathogenic forms at the expense of the harmless saprophytes.

Method.

1. Number a set of Bile salt broth tubes 1-5.
2. Number a set of Bile salt broth tubes 1a-5a.
3. Number one flask 6 and another 7.
4. To tubes No. 1 and 1a add 0.1 c.c. water sample.
To tubes No. 2 and 2a add 1.c.c. water sample.
To tubes No. 3 and 3a add 2.c.c. water sample.
To tubes No. 4 and 4a add 5c.c. water sample.
To tubes No. 5 and 5a add 10.c.c. water sample.
5. Put up all the tubes in Buchner's tubes and incubate aerobically at 42° C.
6. Pipette 25 c.c. of double strength bile salt broth into flask 6 and 50 c.c. double strength bile salt broth into flask 7.
7. Pipette 25 c.c. water sample into flask 6 and 50 c.c. water sample into flask 7.
8. Incubate the two flasks aerobically at 42° C.
9. After the end of 24 hours incubation, note each culture:
 - (a) The presence or absence of visible growth.
 - (b) The reaction of the medium as indicated by the colour change, if any, the litmus has undergone.
 - (c) The presence or absence of gas formation as indicated by a froth on the surface of the medium and

the collection of gas in the inner "gas" tube.

10. Replace those tubes which show no signs of growth in the incubator.

Examine after another period of 24 hours with reference to points indicated above.

11. Remove culture tubes which show visible growth from the Buchner's tubes, whether acid production and gas formation are present or not.
12. Examine all tubes showing growth by hanging-drop preparations. Note such as show the presence of chains of cocci.
13. Prepare surface plate cultivations upon nutrose agar from each tube that shows growth either macroscopically or microscopically and incubate for 24 hours aerobically at 37.5 C.
14. Examine the growth on the plate either with the naked eye or with hand lens. Pick off for subcultivation of the coli group, typhoid group, paratyphoid group and the streptococci. (Practice will facilitate the recognition of the groups).
15. The **coliform** or **typhiform** colonies are streak or smear subcultivated upon nutrient agar and incubated aerobically for 24 hours at 37.5° C.
 - (a) Examine growth of each tube macroscopically and microscopically.

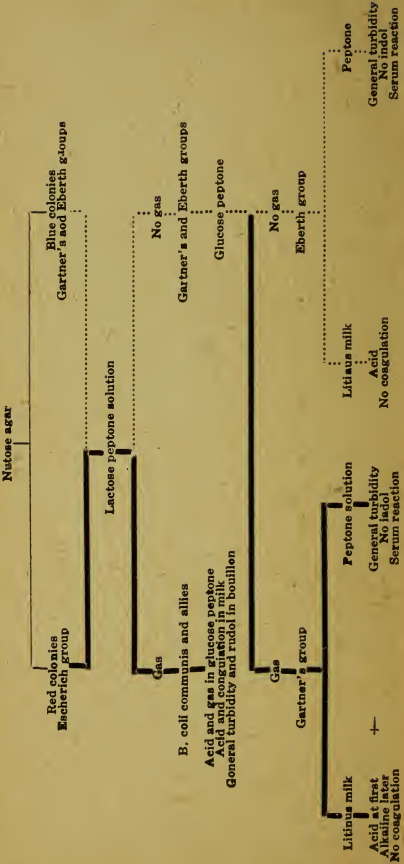
If growth is impure, replate on nutrose agar, pick off colonies and resubcultivate till pure, then add 5 c.c. sterile normal saline or sterile broth and emulsify the entire surface growth with it.

- (b) From the emulsion prepare a series of subcultivations by loop smears on slanted gelatin, slanted agar potato, and by adding 0.1 c.c. of emulsion to nutrient broth, litmus milk, dextrose peptone, levulose peptone, galactose peptone, maltose peptone, saccharose peptone, raffinose peptone, dulcitate peptone, marmite peptone, glycerin peptone, inulin peptone and dextrin peptone.
 - (c) Differentiate the bacilli by means of the cultural and biological characters into:

1. **Escherich group.** *B. Coli communis*, *B. Coli communior*, *B. lactis aerogenes* and *B. Cloacae*.
2. **Gaertner group:** *B. enteritidis* (of Gaertner), *B. paratyphosus* A. B. *paratyphosus* B. and *B. cholerae* suum.
3. **Ebert group:** *B. typhosus*, *B. dysenteriae* (Shiga), *B. dysenteriae* (Flexner), and *B. fercalis alcaligenes*.

(d) Confirm results by specific agglutinating sera obtained from experimentally inoculated animals. If a positive result is obtained by this method, it needs only a simple calculation to determine the smallest quantity of the sample that contains at least one of the organisms of indication. e.g. if growth due to *B. Coli* in tubes from 4 to 10, it follows that at least one colon bacillus is present in every 10 c.c. of the water sample, but not in every 5 c.c.

SCHEME FOR ISOLATION OF MEMBERS OF COLI AND TYPHOID GROUPS



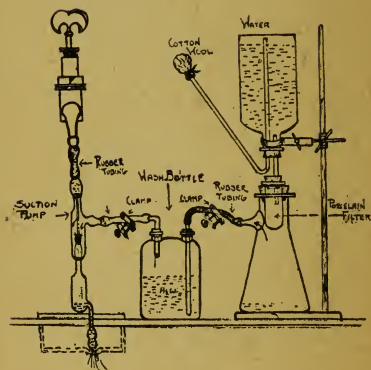
16. **Pick off streptococcus colonies** and subcultivate upon nutrient agar as directed in steps a and b of 15.

Differentiate the streptococci isolated into members of the (a) Saprophytic group — short-chained cocci.

(b) Parasitic (pathogenic) group — long-chained cocci by their cultural characters and record numerical frequency as indicated after — of 15.

Determine the pathogenicity for mice and rabbits of the streptococci isolated.

- B. **Concentration Method.** Organisms in water that are few in number are best sought for by the concentration method. The quantity of water required for this examination is about 2000 c.c.



Method.

1. Fit up filtering apparatus as in the accompanying diagram (after Eyre).
2. Filter the entire 2000 c.c. of water through the filter candle.
3. When filtration is completed, screw up the clamp so as to occlude the two pieces of pressure tubing.
4. Reverse the position of the glass tubes in Wolff's bottle, so that the one nearest the air pump now dips into the H^2SO_4 .
5. Slowly open clamps and allow air to gradually pass through the acid, and enter flask, and so restore pressure.
6. Unship the apparatus, remove the cork from mouth of candle.
7. Pipette 10 c.c. of sterile broth into the interior of the candle, and by means of a sterile test-tube brush emulsify the slimy residue which lines the candle, with the broth.

Practically all of the bacteria contained in the original 2000 c.c. of water will now be contained in the 10 c.c. emulsion of broth, so that 1 c.c. of emulsion is equivalent, so far as the contained organisms are concerned, to 200 c.c. of the original water.

Coli-Typhoid Group.

1. Number 9 tubes of bile salt broth from 1-9.
2. To No. 1 add 1 c.c. of the original water sample before filtration is commenced.
To No. 2 add 2 c.c. of the original water sample before filtration is commenced.
To No. 3 add 5 c.c. of the original water sample before filtration is commenced.
3. To No. 4 add 0.05 c.c. (equivalent to 10 c.c. of original water sample).
To No. 5 add 0.125 c.c. (equivalent to 25 c.c. of original water sample).
To No. 6 add 0.25 c.c. (equivalent to 50 c.c. of original water sample).
To No. 7 add 0.5 c.c. (equivalent to 100 c.c. of original water sample).
To No. 8 add 1.0 c.c. (equivalent to 200 c.c. of original water sample).
To No. 9 add 2.5 c.c. (equivalent to 500 c.c. of original water sample).

4. Put up each tube anaerobically in a Buckner's tube and incubate at 42° C.
5. Subsequent steps are same as those described under enrichment method.

B. Enteritidis Sporogenes.

1. Transfer 5 c.c. of emulsion from the filter to a sterile test-tube and plug carefully.
2. Place test-tube into the interior of a benzole bath, and expose to a temperature of 80° C. for 20 minutes.
3. Number 10 tubes of litmus milk from 1-10.
4. Remove test-tube from benzole bath and shake well to distribute spores through fluid.
5. Add to each tube of litmus milk a measured quantity of suspension *vide coli* group.
6. Incubate anaerobically at 37.5° C. (Put up in Buchner's tubes or in Bulloch's apparatus, or pour layer of sterile vaseline on surface of fluid).
7. Examine after 24 hours.
 - (a) Acid reaction.
 - (b) Presence of clotting and separation of clear whey.
 - (c) Presence of gas.
8. Replace tubes showing no signs of growth in incubator for another 24 hours and again examine *vide* 7.
9. Remove tubes showing growth, carefully pipette off whey, and examine microscopically.
10. Inoculate 2 guinea-pigs subcutaneously with 0.5 c.c. of whey and observe result.

Vibrio Cholera.

1. Number ten tubes of peptose water from 1-10.
2. To each tube add a measured quantity of emulsion, *vide coli* group.
3. Incubate anaerobically at 37.5° C. for 24 hours and examine for delicate pellicle formation, which if present, examine microscopically.
4. Inoculate fresh tubes of peptone water from tubes showing pellicle formation and incubate for 24 hours.
5. Test peptone water for nitrol and nitrite.

6. Pick off colonies resembling cholera colonies and subcultivate on all ordinary media.
7. Test vibrio isolated against serum of an animal immunized to cholera for agglutination.

B. Anthrax.

- 1 and 2 vide B. enteritidis sporagenes.
3. Inoculate a young white rat subcutaneously with 1 c.c. of emulsion. Observe during life, if animal dies, make post mortem examination.
4. Make nutrient agar plates with 0.2 c.c., 0.3 c.c., and 0.5 c.c. quantities of suspension and incubate at 37.5° C., for 24 or 48 hours.
5. Pick off anthrax-like colonies and subcultivate on all ordinary media.
6. Inoculate another white rat as in 3, using 2 loopfuls of agar subcultivation emulsified with 1 c.c. sterile bouillon. Observe as in 3.

B. Tetani.

1. Vide 1 and 2 of B Anthrax.
2. Add 1 c.c. of suspension to each of 3 tubes of glucose formate broth, incubate anaerobically in Buchner's tubes at 37.5° C.
3. From such tubes showing visible growth after end of 24 hours incubation, inoculate guinea-pigs subcutaneously, using 0.1 c.c. of bouillon cultivation. Observe vide 3 B Anthrax.
4. From the same tubes pour agar plates and incubate anaerobically in Bulloch's apparatus at 37.5° C.
5. Subcultivate suspicious colonies on various media, incubate anaerobically, making controle cultivation on glucose formate agar, stab and streak, to incubate anaerobically and carry out further inoculation experiments with resulting growths.

Interpretation of bacteriological water analysis.

In the analysis of water, data, such as the kind of water, the method of collection, the sampling, rain fall, transmission, etc., must be recorded in order that the results may be properly interpreted. Several analyses are necessary and should be made regularly and systematically.

The number of micro-organisms permissible in potable water depends to a great extent upon the kind of micro-organisms présent. Great numbers of bacteria indicate a large amount of organic matter. The number of bacteria in deep wells and springs should not exceed 50 per cc. on gelatin at 20° to 22° C. Organisms in excess of the above figures would indicate pollution, except after rains or floods.

The number of organisms grown on agar at a temperature of 37½° C. is probably more important than the number present in the "gelatine count" in as much as many water bacteria do not grow at this heat, whereas the sewage and soil organisms grow very rapidly at 37½° C. The agar count would therefore eliminate the water flora, but would obscure the sanitary results by reason of the presence of soil organisms.

The agar count of deep waters should not exceed 10 per cc. and for surface water it should not exceed 100 per cc.

Isolation and identification of specific disease organisms from water would condemn such a water as unfit for use; but by reason of the difficulty of such an examination their isolation is not often attempted.

The isolation of the colon bacillus from water is easily carried out and its presence is generally looked upon as significant and indicative of sewage pollution. The number of bacilli coli in a certain amount of water sufficient to condemn it varies in the opinion of different authorities. Prescott and Winslow hold that if present in 1 cc. of water it is reasonable proof of serious pollution. Savage suggests that the bacillus coli should be absent from 10 cc. in surface waters, such as rivers used for drinking purposes, shallow wells and upland surface waters.

The streptococcus is also an indication of sewage contamination, and should be absent from the amounts of water mentioned above for the bacillus coli.

The bacillus enteritidis sporogenes should not be present in 1,000 cc. water from deep wells nor in 100 cc. from surface waters.

THE BACTERIOLOGY OF SEWAGE.

Sewage is a menace to the public health because of the frequent presence of pathogenic bacteria. It is made up of the products of man and animal waste. A constant or characteristic bacterial flora cannot be established. A classification based upon bacterial activity rather than upon the species, the genus, the group or type has been adopted by reason of the organism's activity in sewage purification.

Certain exceptions to these general principles are taken in case of such organisms as the bacillus coli, the sewage streptococcus and the bacillus enteritidis. These are to a certain extent characteristic sewage bacteria and their interest in them as individuals has to do with water bacteriology.

According to the general character of changes which the sewage organisms bring about they are divided into two large groups as follows:

- (1) **The anærobic or putrefactive bacteria** which bring about the withdrawal of oxygen from one molecule or part of a molecule and the subsequent oxidation of another molecule or part of the same molecule. The energy released in this process is utilized in the vital functions of the organisms. It involves the reduction of urea, the hydrllis of protein, and of cellulose, the emulsification of fats, the reduction of nitrates and sulphates and possibly phosphates.
- (2) **The Oxidizing Bacteria.** They are distinguished by the fact that oxygen is added to the molecule, the product containing more oxygen than the initial substance. Carbon dioxide, water and nitrates are produced in distinction from methane hydrogen and ammonia which characterizes the anærobic reactions.

Pathogenic Bacteria. It is assumed that they are always present in sewage. Sewage can be made harmless by being sterilized but can be freed from offense only by the destruction of organic matter, and this is obtained almost wholly

through bacterial processes, except when chemical precipitants are used.

There are two general methods employed for the cultivation of those bacteria which are of assistance in sewage purification. They may be cultivated in filters of sand or coarser material, or in specially constructed tanks called "septic tanks."

Septic Tank Purification.

Cameron, 1895, introduced the "septic tank." In this tank, the sewage was admitted at the bottom and flowed out at the top after about 24 hours' subjection to anærobic conditions acting upon the organic matter as indicated above. Soil and sand filters act not only mechanically, but also bacteriologically and offer one of the best means of purifying sewage bacteriologically. Sewage is conducted to beds, allowed to pass through and then after a few hours again poured on. This purification is produced by the action of ærobic bacteria. The best results are obtained by combining the two processes; first, the anærobic treatment to break down the solid material, then the sand filtration to oxidize the compounds and render these products harmless.

The biological processes remove bacteria not by any specific antagonistic action but by delaying their passage and permitting the natural decrease that occurs when multiplication is prevented.

Sewage Analysis.

On account of the great numbers of bacteria in sewage it becomes necessary to make dilutions ranging from 1-1000 to 1-10000 and then plating, etc., as in water analyses.

In sewage chemistry, putrefaction is that change which takes place naturally in sewage after anærobic conditions have become established. It involves the reduction of urea, the hydrolysis of protein and of cellulose, the emulsification of fats, the reduction of nitrates and sulphates and possibly of phosphates and those other changes which are characterized by the withdrawal of O and the hydrolysis of complex molecules. These changes are always noted in sewage under anærobic conditions, and the terms putrefactive and anærobic change are for the present purposes practically synonymous.

BACTERIOLOGY OF SOIL

Many varieties of bacteria are present in the soil. Some by reason of contamination through animal feces and other waste products, but the majority are real soil bacteria in that they live and multiply chiefly in the soil. They have important functions to perform in continuing the earth's supply. Some of the bacteria make carbon, nitrogen, hydrogen and other compounds locked up in the dead bodies of animals and plants available for plants. Other bacteria manufacture food for plants from the gases of the air and the inorganic elements of the earth, which in their simple forms were not available. They, therefore, form an important link in the earth's life cycle. Food, moisture and proper temperature are necessary for their activities.

In a grain of rich loam there may be many millions, while an equal quantity of sand may be almost free from bacteria.

Various species of soil bacteria have an influence upon each other. The anaerobic bacteria are enabled to develop by the aerobic species utilization of the free oxygen, while still other species make assimilable substances which cannot be used by others.

Carbon compounds are broken up by the soil bacteria. Starch is manufactured by plants then converted into cellulose, wood fats and sugar, which when formed, cannot be utilized by other plants.

The largest part of these substances are broken up by micro-organisms; a smaller portion are transformed within the animal body. Alcohol is fermented from sugars and starch by the yeasts and molds with the production of carbon dioxide, or acid fermentation by the action of bacteria takes place, with the production of acids and often of carbon dioxide.

Cellulose is attacked by certain varieties of bacteria, acting both in the presence and absence of free oxygen. Moulds also act on cellulose with production of carbon dioxide, gas and other products. Wood is first attacked by fungi, then by bacteria.

Animals utilize plant proteids and reduce them to simpler compounds as

urea, etc., but these compounds are not suitable for plant use, so that micro-organisms must break these compounds into more simple form. Yeasts, molds and fungi decompose these substances (plant proteids) to a certain extent, but the chief decomposition is carried out by bacteria.

In the absence of oxygen, the process is incomplete with the production of H_2S , NH_3 and CH_4 , and is termed putrefaction. The presence of oxygen gives rise to more complete decomposition with the production of CO_2 , N and H_2O .

The variety of organisms producing these changes are many. Some are found in decaying vegetable matter, others in animal tissue.

There is a process of oxidation produced by bacteria, in which ammonia compounds are changed to nitrates and thus utilized by plants (nitrification). The ammonia is first oxidized to nitrite, then into a nitrate, which is taken up by the plant roots from the soil. The two organisms isolated causing a change of ammonia to nitrites, are the nitrosomonas and the nitrosococcus. One variety of organism changing nitrites to nitrates is called nitrobacter. These organisms appear to depend upon mineral substances for their food. A small amount of organic matter in the media acts as antiseptics. Plants take up most of their nitrogen in the form of nitrates; hence these bacteria are important. When the soil becomes acid, the growth ceases. Air is necessary for their action as the process is one of oxidation.

There is also a reduction process called denitrification. Nitrates yield a part or all of its oxygen and becomes changed to nitrites, ammonia, and free nitrogen.

In the partial change, the soil does not lose its available nitrogen which takes place in the total change, by changing nitrites and ammonia by the nitrifying bacteria to nitrates.

The types of nitrogen reduction are:

1. The reduction of nitrates to nitrites and ammonia.
2. The reduction of nitrates and nitrites to gaseous oxides of nitrogen.
3. The reduction of nitrites with the development of free nitrogen.

Certain plants are able to use the nitrogen of the air through the aid of bacteria growing in and producing enlargements (tubercles), on the roots. The root bacteria are called *B. radiculicola* and may remain active in the soil for long periods even though there is no leguminous vegetation.

The organism diffuses rapidly in soils that are in proper condition, so that if a soil lacks the organism, it cannot be introduced to it until the soil has been made fit for the organisms development. Buchanan concludes that:

1. The *B. radiculicola* varies considerably in its morphology when appropriate nutrients, as the salts of organic acids, are induced into the artificial media. Sodium succinate produces a most luxuriant growth together with the greatest variety of bacteroids.
2. The *B. radiculicola*, in the roots of legumens, may show the same type of bacteroids as seen in suitable artificial media, and again the same type may not be the same as produced in culture-media and that produced in the nodule by the same form.
3. The *B. radiculicola* probably includes a group of closely related varieties or species which differ from each other and morphological characters.
4. The organism of the nodule resembles morphologically both the yeasts and the bacteria. The difference between this form and the forms included under *Bacillus* and *Pseudomonas* justify the generic use of a separate generic name of *Rhizobium*.

Winogradsky states that certain anaerobic, spore-bearing, bacilli (*Clostridium Pasteurianum*) outside of the roots perform the same function as those within the roots. Their power of nitrogen fixation is increased in the presence of sugar and decreased in the presence of nitrogenous substances.

Beyerinck and Bailey have described aerobic species of nitrogen fixing bacteria, to which the name of *Azotobacter* has been given.

The inoculation of soils and an investigation of soils and crops best fitted

for the growth of these bacteria has been carried out with the result of greatly improving impoverished soils.

The use of seeds inoculated with a special variety of bacteria suitable for the plant and soil has been largely practiced with marked results. Excessive bacterial development may at times be harmful to the soil.

The exhaustion of the soil following the constant raising of the same crop is now thought to be due partly to the inability of a few restricted species of bacteria, continued in the soil, to produce the substances necessary for the nutrition of the special crop, or that the bacteria use up the substances in the soil necessary to crop growth.

The greatest number of bacteria are found a little below the surface of the soil.

Some of the bacterial products act upon the inorganic constituents of the soil. The CO_2 and organic acids act upon the compounds of lime and magnesia, and convert them into more soluble substances. The same is true of the rock phosphates, the silicate of potassium, sulphates, etc.

Quantitative Analysis of soil for bacteria

Include 4 distinct investigations:

1. Enumeration of Aerobic organism.
2. Enumeration of Spores of Aerobes.
3. Enumeration of Anaerobic organisms (also facul. Anaerobes).

4. Enumeration of Spores of organ.

Further by a combination of results of 1-2 and 3-4, the ratio of spores to vegetative forms is obtained.

1. Obtain soil under sterile conditions.
2. Weigh and make proper dilutions for counting.

3. (A) **Aerobe**—

Pour set of gelatin plates.

Incubate at 20°C .

Pour set of agar plates.

Incubate at $37\frac{1}{2}^\circ \text{C}$.

4. Count plates for 3, 4 or 5 days.

(a) The number of aerobic micro organisms per 1 gm. soil.

(b) The number of yeast and moulds per 1 gm. soil.

(c) The number of aerobe growing at 37°C ., per 1 gm. soil.

- (B) **Anaerobes** spores and Veg.
 Pour set of plates in glucose.
 Formate gelatin and agar.
 Incubate in Bulloch's apparatus.
- (C) **Aerobes and Anaerobes (spores only).**
- (1) 5 c.c. of soil dilution in sterile tube.
 - (2) Differential sterilize at 80 for 10 minutes.
 - (3) Pour plates and incubate anaerobically.
 - (4) After long incubate, count.

Qualitative—for

Coli group—Typhoid group.

B. Anthrax, B. Tetanus, B. Malig Oedema.

Nitrous Organism, Nitric Organisms.

Nitrous Organ.

10 tubes of **Winogradsky's Sol. No. 1.**
 Label from 1-10.

Inoculate each tube with varying dilutions and incubate at 30° C.

Nitric Organisms.

10 tubes of Winogradsky's Sol. No. 11, and incubate as in 1.

Incubate at 30° C.

Examine after 24-48 and from those tubes that show signs of growth make subcultivations in fresh tubes of same media and incubate at 30° C. Make further subcultivation from these and again incubate.

If growth occurs in these sub-cultures, make surface smears on plates of Winogradsky's silicate Jelly. Pick off colonies as make appearance and sub-cultivate in each of these two media.

W. Sol. for Nitric.

1. K. Phosphate, 1 gm.

Mg. Sulph., .5 gm.

Ca. Chloride, .01 gm.

Na Chlor, 2 gm.

Dissolve in A.D., 1000 c.c.

2. Fill into flasks in quantities of 20 c.c., and add to each a small quantity of freshly washed mg. Carb.
3. Sterilize in steamer at 100 for 3 days.
4. Add to each flask, 2 c.c. of sterile 2% sol Ammonia Sulph.
5. Incubate at 37° for 48 hours and eliminate any containing a growth.

The Media for the growth of Nitros Organisms.

1. Ammon. Sulph., 1 gm.
 K. Sulph., 1 gm.
 A.D., 1000 c.c.

2. Add 5-10 gm. basic mg. Carb (sterilize by boiling).

3. Fill flasks and sterilize as in No. 1.

W. Silicate Jelly.

Sol. A.

Ammon Sulph., 40 gm. Mg.
Sulph., 0.05 gm.

Calcin Chloride, .01 gm., A.D.
50 c.c.

Sol. B. K. Phosphate .10 gm. Na Carb
.60 gm. A.D. 50 c.c.

Silica acid 3.4 gm., A.D. 100 c.c.

Pour them into a large dish, (Porcelain).

5. Mix of Sol. A & B then add successive small quantities of mixed salts to silicic acid sol. (stir const.) with glass rod till a Jelly of right consistency is found.

6. Spread layer of Jelly over several Petri dishes.

Sterilize for 30 minutes on 3 days.

THE BACTERIOLOGY OF AIR.

The atmosphere is not the normal habitat of bacteria. Their growth and multiplication can not take place in it under ordinary conditions. The air is kept in motion by the wind, so that fine particles are constantly being carried into it from the ground, especially so in inhabited areas. The bacteria in the dust of the field and street are carried along with the dust particles of the air and are usually of the harmless soil variety or the almost harmless intestinal bacteria of animals. Pathogenic human bacteria are rarely carried in harmful numbers except under exceptional circumstances, and are usually in form of spores, e. g., anthrax bacillus, tetanus bacillus. On a dry, windy day the air contains many thousands of bacteria per cubic meter. In warm weather the rain carries down the bacteria of the air. After a storm there are very few bacteria present in the air. The bacteria in the air of the country are much less than the bacteria in the air of the cities. Forests decrease the number of bacteria. Bacteria are very few on high mountains; also on the high seas. The bacteria that multiply in the streets and in the soil are almost always saprophytic. The bacteria present in the air of dwellings depend upon factors such

as the opening of windows to the outside, the cleanliness of the dwelling and the stirring up of dust by sweeping. It is nearly impossible to separate the effects of the bacteria which we inhale from that of the dust particles which they accompany. Both probably act as slight irritants and so predispose to definite infections.

It is problematical as to the importance of air as a means of conveying disease, though there can be no doubt that smallpox, measles, scarlet fever, etc., are transmitted readily, and pulmonary anthrax and tuberculosis, pneumonia, influenza, diphtheria and meningitis may result from inhalation of the organisms.

The distance through which the air may carry the causative agents of disease requires further study and must necessarily depend upon a variety of conditions, as time, degree of moisture, air currents, factors producing desiccation, effects of sunlight, etc.

Bacterial Air Examination.

Gelatin or agar plates may be exposed to the air for a definite length of time and then incubated at both 25° and $37\frac{1}{2}^{\circ}$ C. temperatures.

The number of colonies appearing on the plates will indicate in a general way the bacterial content of the air. The results must necessarily vary according to the degree of moisture in the atmosphere, air currents, etc., and therefore furnishes no standard for comparative results.

A method in use at the present time, from which more accurate results may be obtained, follows, vide:

1. Fill 10 litres of water into an aspirating bottle.
2. Construct a sand filter from a small glass tube containing about 4 c.m. depth of quartz sand held in place by means of a wire screen. Sterilize in hot air oven.
3. Insert sand filter in a perforated rubber stopper which fits mouth of aspirating bottle.
4. By allowing water to flow from lower opening of aspirating bottle,

10 litres of air is now drawn through the sand filter. (Refill aspirating bottle and aspirate as many times as necessary to give the quantity of air required for the test.)

5. Remove the sand filter and carefully pour the sand into a known quantity of sterile bouillon or water.
6. From the bouillon-sand mixture (which contains in suspension the total number of bacteria contained in the quantity of air aspirated through the sand) plate 1 c.c. in gelatin or agar and from the colonies appearing thereon estimate the number of bacteria contained in a given quantity of air.

By the use of the aspirating bottle the air may be drawn directly through a measured quantity of sterile bouillon or water in an Erlenmeyer flask as follows:

- (1) Erlenmeyer flask of 250 c.c. capacity containing 50 c.c. Bouillon or water.

- (2) Rubber stopper to fit mouth of flask perforated with 2 holes and fitted as follows: Take a 9 c.m. length of glass tubing and bend up 3 c.m. at one end at right angles to main length. Pass long arm of the angle through one of the perforations in the stopper. It must not come in contact with the bouillon.

Take a glass funnel 5 or 6 c.m. in diameter, with a stem 12 c.m. in length, and bend stem close up to the apex of the funnel, in a gentle curve through a quarter of a circle; pass the long stem through the other perforation in the rubber stopper. Make sure the end of the stem of the funnel is immersed in the bouillon.

- (3) Sterilize flask, contents and fittings.

- (4) Attach aspirating bottle to small glass tube and operate as in "4" of sand filter.

- (5) Make plates from bouillon as in "6" of sand filter.

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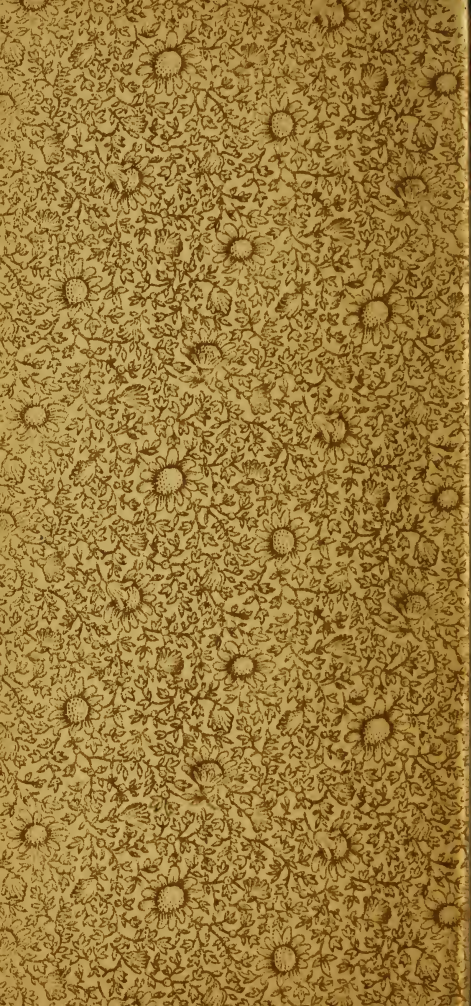
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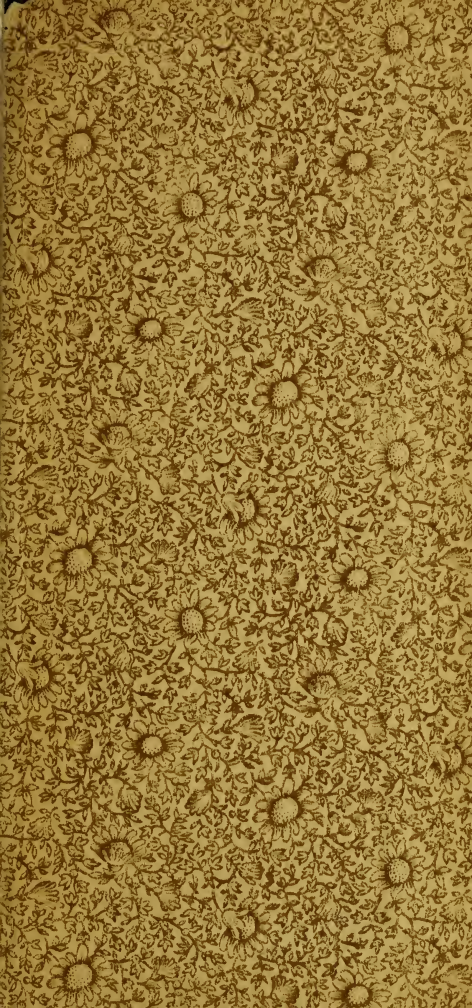
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